BIOCHEMICAL AND MUTANT ANALYSIS OF NITRITE REDUCTION IN BARLEY

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at the
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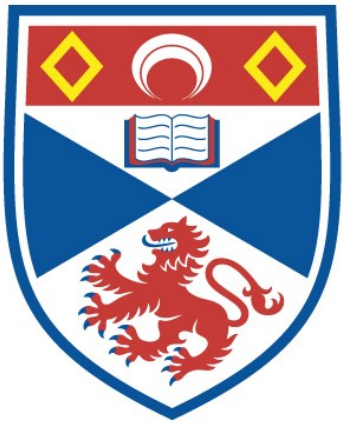

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BIOCHEMICAL AND MUTANT ANALYSIS OF NITRITE REDUCTION IN BARLEY

by

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ABSTRACT

The object of this study was to isolate and characterise barley mutants that lacked a functional nitrite reductase activity. This work should complement previous studies on nitrate reductase to develop a fuller understanding of nitrate assimilation in barley.

30 nitrite reduction-deficient M_2 barley plants (azide-treated in the M_1) were identified as nitrite accumulators after treatment with nitrate. Biochemical analysis of M_2 selections revealed that leaf tissue from 9 selected plants lacked detectable nitrite reductase protein.

Progeny from 4 of the selected plants (Golden Promise 2406, Tweed 3999, Klaxon 1010 and Klaxon 2760) inherited the phenotype of a lack of leaf nitrite reductase protein. These plants also lacked significant *in vitro* nitrite reductase activity. However, *in vitro* nitrate reductase and nitrate accumulation were comparable with wild type controls.

Root tissue from nitrate-treated progeny of Golden Promise 2406, Tweed 3999, Klaxon 1010 and Klaxon 2760 also lacked nitrite reductase protein.

Loss of nitrite reductase protein in selection Tweed 3999 was caused by a single recessive nuclear gene mutation.

Thus, these plants are defective in nitrite reduction due to the inherited loss of nitrite reductase protein molecules in both leaf and root after treatment with nitrate in the light. This defect is caused by a mutation within a single nuclear gene in selection Tweed 3999.

Analysis of wild type barley cv. Golden Promise revealed that increases in nitrite reductase activity in response to treatment with nitrate and light in leaf tissue and with nitrate in root tissue are due to *de novo* synthesis of enzyme molecules.

In situ immunogold labelling of barley leaf sections with nitrite reductase antiserum demonstrates that the majority of labelling occurs within the chloroplasts.

Contents

ACKNOWLEDGEMENTS

I would like to thank Dr. John Wray for his advice and criticism throughout this work. I would also like to express my thanks to Mrs. Amanda Gilkes for her work in screening M_1 spikes for nitrite accumulators and her help in maintaining selected plants in hydroponic culture. My thanks go also to Mrs. Anne Holyoake and Miss Amanda Wilshin for their work in screening bulk-harvested M_2 seed for nitrite accumulators and Mr. Denis Kirk for his help in harvesting M_1 barley spikes.

I would like to thank Dr. Roger Wallsgrove, Rothamsted Experimental Station, for providing bulk-harvested M_1 seed and the production and growth of M_1 barley plants from which M_1 spikes were harvested. I would also like to thank Dr. William Thomas, Scottish Crop Research Institute, for providing growing facilities for M_1 populations of barley from which M_1 spikes were harvested.

^{15}N analysis was performed at Rothamsted Experimental Station by David Powlson and Paul Poulton.

DECLARATION

I, Euan Duncanson, hereby certify that this thesis has been composed by myself, that it is a record of my own work, and that it has not been accepted in partial or complete fulfilment of any other degree or professional qualification.

August 1990

ACADEMIC RECORD

I was admitted to the Faculty of Science of the University of St. Andrews under Ordinance General No 12 on 1st October 1986 and as a candidate for the degree of Ph.D. on 1st October 1986.

August 1990

CERTIFICATE

I hereby certify that the candidate has fulfilled the conditions of the Resolution and Regulations appropriate to the Degree of Ph.D.

August 1990

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Abbreviations

cv.	: cultivar
GP	: Golden Promise
IX	: Klaxon
IT	: Tweed
IN	: Natasha
IV	: Vista
DO	: Doublet
CO	: Corniche
DI	: Digger
F_1	: Population derived from cross pollinations between selected M_2 nitrite accumulators and wild type plants
F_2	: Generation derived from self pollination of F_1 population
M_1	: Population treated with chemical mutagen
M_2	: Generation derived from self pollination of M_1 population
M_3	: Generation derived from self pollination of M_2 population
M_4	: Generation derived from self pollination of M_3 population
NiR	: Nitrite reductase
NR	: Nitrate reductase
CRM	: Cross reacting material
PAGE	: Polyacrylamide gel electrophoresis
BCIP	: 5-bromo-4-chloro-3-indolyl phosphate
BSA	: Bovine serum albumin
EDTA	: ethylenediaminetetra-acetic acid
NaFe EDTA	: ethylenediaminetetra-acetic acid, ferric monosodium salt
FAD	: Flavin adenine dinucleotide
GAR G15	: Goat anti-rabbit IgG-gold conjugate (15nm)
MES	: 2[N-morpholino]ethanesulphonic acid
MV	: Methyl viologen (1,1'-dimethyl-4-4'-bipyridinium chloride)
NADH	: β-nicotinamide adenine dinucleotide, reduced form
NADPH	: β-nicotinamide adenine dinucleotide phosphate, reduced form
NBT	: Nitroblue tetrazolium (diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride

NED	: N-1-naphthylethylenediamine dihydrochloride
SDS	: Sodium dodecyl sulphate
Szechrome NAS®	: Diphenylamine sulphonic acid chromogene
TEMED	: N,N,N',N'-tetramethyl-ethylene diamine ethane

Chapter 1

Introduction

1.1. Introduction

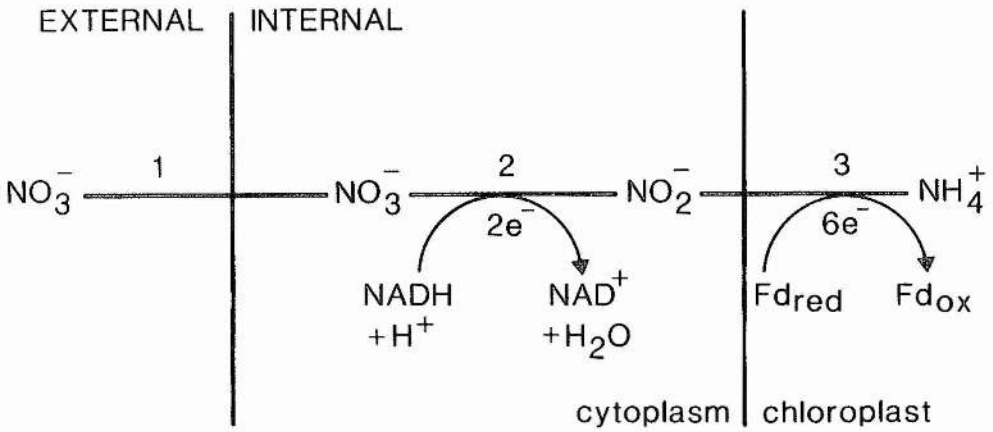
1.1.1. Nitrate assimilation

Nitrate is the major nitrogen source for most cultivated crop plants grown under normal field conditions. Although in natural ecosystems ammonium as well as nitrate is formed by mineralisation of organic soil nitrogen, and there is considerable input of ammonium fertiliser nitrogen in agricultural ecosystems, the oxidation of ammonium by autotrophic nitrifying bacteria such as *Nitrosomonas* and *Nitrobacter* species ensures that in most well-aerated soils inorganic nitrogen is mainly available to the plant as nitrate (reviewed in Haynes, 1986).

Nitrogen and carbon constitute approximately 2 per cent and 40 per cent respectively of the dry weight of plant material. On a worldwide scale $200x10^9$ tons of carbon are fixed annually (Galston, 1961) which on a basis of approximate analysis would require the incorporation of $10x10^9$ tons of nitrogen per year. Biological dinitrogen fixation accounts for only a small proportion of nitrogen incorporation with the total, annual amount of dinitrogen fixed in the biosphere estimated at $0.175x10^9$ tons (Burns and Hardy, 1975). Thus, the majority of nitrogen incorporated into plant material occurs through the production and subsequent assimilation of nitrate.

Nitrate assimilation, defined here as the uptake of nitrate into the plant and its subsequent two-step reduction to ammonium ions by the enzymes nitrate reductase and nitrite reductase, requires eight electrons (Fig 1.1). In non-chlorophyllous tissue these eight electrons are derived from carbohydrate oxidation (Beevers and Hageman, 1980). In chlorophyllous tissue two electrons are derived from carbohydrate oxidation while six are derived from the trapping of light energy (via ferredoxin) (Beevers and Hageman, 1980). The reduction of carbon dioxide to the level of carbohydrate requires four electrons. Higher plants utilise, on average, about 25 per cent of the energy required for the fixation of carbon dioxide on the assimilation of nitrate (Guerrero *et al.*, 1981). The ammonium produced must then be incorporated into organic compounds converting ammonium-nitrogen into α-amino-nitrogen. Thus, the overall assimilation of nitrate into α-amino-nitrogen requires a massive amount of energy.

Fig 1.1. Nitrate assimilation pathway



1: nitrate uptake
2: nitrate reductase
3: nitrite reductase

1.1.2. Nitrate uptake

Nitrate uptake, its subsequent allocation to storage, transport or reduction and its remobilisation within the plant are the most poorly characterised steps of the nitrate assimilation pathway. Uptake into the root is likely to be a carrier-mediated process utilising metabolic energy since it occurs against an electrochemical potential gradient, with anaerobic conditions (Trought and Drew, 1981), uncouplers of oxidative phosphorylation (Rao and Rains, 1976) and low temperature (Clarkson and Warner, 1979) being inhibitory.

Nitrate uptake rates follow a dual-phase relationship with the concentration of nitrate available to the plant. Both phases appear to be hyperbolic in accordance with Michaelis-Menten kinetics and suggest the existence of at least two uptake mechanisms operating at high and low nitrate concentrations respectively (Rao and Rains, 1976; Doddema and Telkamp, 1979). One system is likely to be "constitutive" and operates in plants which have not been exposed to nitrate, while the other is an "inducible" system and is characterised kinetically by experiments which show that maximal rates of net uptake occur after a lag of from one to a few hours after exposure of roots to nitrate (Jackson *et al.*, 1986).

The development of maximal rates of net nitrate uptake from ambient solutions by roots is prevented by inhibitors of RNA and protein synthesis (Tompkins *et al.*, 1978; Rao and Rains, 1976) suggesting that perhaps some component of the induced nitrate uptake system is synthesised *de novo* in response to the presence of nitrate.

1.1.3. Nitrate reductase

The nitrate reduction step is without doubt the best characterised step in nitrate assimilation at the physiological, biochemical and molecular level and has been the subject of numerous reviews (Hewitt, 1975; Beevers and Hageman, 1980; Guerrero *et al.*, 1981; Campbell and Smarelli, 1986; Wray, 1988).

Higher plant nitrate reductases are flavohaemomolybdoproteins which catalyse the two electron reduction of nitrate to nitrite (Hewitt and Notton, 1980). Almost all higher plants so far examined possess NADH-nitrate reductase (EC 1.6.6.1.) which has a pH optimum around 7.4 and a Michaelis constant for nitrate and NADH of about 200μM and 2μM respectively (Beevers and Hageman, 1980; Guerrero *et al.*, 1981). Some plants also possess a bispecific NAD(P)H-nitrate reductase (EC 1.6.6.2.) which uses either NADH or NADPH as electron donor (Shen *et al.*, 1976; Campbell, 1978; Orihuel *et al.*, 1980).

Biochemical and physiological evidence suggests that NAD(P)H-nitrate reductases are distinct species from NADH-nitrate reductases, possessing a higher Km for nitrate (4mM) and a lower pH optimum (6.5) (Campbell, 1976). The two forms can be separated by chromatography on blue dextran Sepharose (Redinbaugh and Campbell, 1981) and show different developmental (Orihuel-Iranzo and Campbell, 1980) and induction patterns (Shen *et al.*, 1976). Most work has been carried out on the NADH-nitrate reductases and this is outlined below.

Attempts to purify the enzyme in its native state have been made difficult by its extreme sensitivity to proteolytic modification (Brown *et al.*, 1981; Wray and Kirk, 1981; Campbell and Wray, 1983). Estimated molecular weights of the holoenzyme vary from about 200,000 for spinach (Notton and Hewitt, 1979), tobacco (Mendel and Muller, 1980), barley (Small and Wray, 1980) through 220,000 or 230,000 for barley (Kuo *et al.*, 1980) or squash (Redinbaugh and Campbell, 1985) to 270,000 for spinach (Nakagawa *et al.*, 1985).

Nitrate reductase is usually assumed to be a homodimer with subunit molecular weights of 100,000 (Campbell and Wray, 1983) or 110,000 (Kuo *et al.*, 1982) in barley, 105,000 and 114,000 (Nakagawa *et al.*, 1985) or 110,000 and 120,000 (Fido and Notton, 1984) in spinach, and 115,000 in squash (Redinbaugh and Campbell, 1985).

1.1.3.1. Prosthetic groups

Nitrate reductase contains flavin adenine dinucleotide (FAD) (Hewitt and Notton,1980; Redinbaugh and Campbell, 1985). The activity of nitrate reductase is stimulated by exogenous FAD (Maretski *et al.*, 1967; Schrader *et al.*, 1968) suggesting that the flavin is readily dissociable. Purified nitrate reductase from spinach (Notton *et al.*, 1977), tobacco (Mendel and Muller, 1980), barley (Somers *et al.*, 1982) and squash (Redinbaugh and Campbell, 1985) have spectra indicative of the presence of a b-type cytochrome (cytochrome b_{557}). The first definitive evidence for the presence of molybdenum was obtained with the spinach enzyme (Notton and Hewitt, 1971) although it had previously been shown that in barley a non-functional form of the enzyme was synthesised in the presence of the molybdenum analogue, tungsten (Wray and Filner, 1970).

Prosthetic group stoichiometry in squash suggests the presence of one FAD, one haem and one molybdenum per 115,000 molecular weight subunit (Redinbaugh and Campbell, 1985). Electron flow from NADH is generally accepted to be via flavin and haem to

molybdenum which acts as the terminal electron donor to nitrate (reviewed in Hewitt and Notton, 1980).

Molybdenum is carried as a molybdenum cofactor (MoCo) which is a complex between molybdenum and a phosphorylated pterin, molybdopterin (Johnson and Rajagopalan, 1982). The pterin acts as a chelator of molybdenum, interfacing it to the protein and conferring on it biological activity and is also responsible for dimerisation of the protein subunits (Ketchum *et al.*, 1970).

1.1.3.2. Catalytic activities associated with the nitrate reductase holoenzyme

In addition to the overall physiological reaction (NADH-dependent nitrate reduction) the higher plant enzyme carries a dehydrogenase (diaphorase) function usually assayed as cytochrome c reductase as well as flavin mononucleotide and reduced viologen dye nitrate reductase activities. These partial activities are considered to be catalysed by specific regions of the nitrate reductase molecule (Wray and Fido, 1990).

Studies show that the dehydrogenase activity is catalysed by the proximal part of the electron transport chain, perhaps encompassing the flavin domain (Brown *et al.*, 1981) and is an activity of the flavohaemoprotein subunit, independent of the nitrate binding site (Wray and Fido, 1990).

Electrons from flavin mononucleotide and probably from reduced viologen dyes are donated to a later, but undefined, site in the electron transport chain. Since nitrate reductase partial activities are molybdenum-dependent, flavin mononucleotide and reduced viologen dye nitrate reductase activities are functions of the distal part of the electron transport chain.

1.1.3.3. Location

Nitrate reductase is generally accepted to be located in the cytoplasm (Ritenour *et al.*, 1967; Dalling *et al.*, 1972), although it has been proposed that the enzyme was localised within peroxisomes (Lips and Avissar, 1972) or within the chloroplasts (Kamachi *et al.*, 1987).

1.1.3.4. Environmental control

In general, nitrate reductase activity is high in cells or plants grown on nitrate in the light (Beevers and Hageman, 1969; Gupta and Beevers, 1983). While nitrate application leads to increases in nitrate reductase levels nitrate is not an obligatory requirement since several species such as soybean (Lahav *et al.*, 1976) and tobacco (Muller, 1983; Buchanan and Wray, 1982) possess considerable nitrate reductase activity even if never exposed to nitrate.

The level of nitrate reductase activity in leaf tissue is influenced by the intensity of illumination (Sanderson and Cocking, 1964a; Beevers et al., 1965), with nitrate reductase activity remaining at basal levels in pea leaf tissue treated with nitrate in the dark (Gupta and Beevers, 1983). Immunological studies (Somers *et al.*, 1983) show clearly that the increase in nitrate reductase activity seen after nitrate treatment of plants in the light is due to *de novo* synthesis of nitrate reductase molecules. Studies using cDNA species, encoding the nitrate reductase apoprotein gene, as a hybridisation probe show that the steady state level of the nitrate reductase apoprotein mRNA increases markedly (over 100-fold) after nitrate application (Cheng *et al.*, 1986; Crawford *et al.*, 1986), demonstrating that nitrate brings about its effect at the mRNA level. However it is, as yet, unclear whether nitrate regulation occurs at the level of transcription, or acts to modify the stability of the mRNA transcript.

1.1.4. Nitrite reductase

The ferredoxin:nitrite oxidoreductase (EC 1.7.7.1.) (nitrite reductase) enzyme catalyses the six-electron reduction of nitrite to ammonium ions in the third step of the nitrate assimilation pathway (Fig 1.1). Nitrite reductase has been purified to apparent homogeneity from leaf tissue in a number of species including spinach (Ho and Tamura, 1973; Ida and Morita, 1973; Ida *et al.*, 1976; Vega and Kamin, 1977; Ida, 1977; Hirasawa and Tamura, 1980; Ida and Mikami, 1986), *Cucurbita pepo* (Hucklesby *et al.*, 1976), barley (Serra *et al.*, 1982; Ip *et al.*, 1990), wheat (Small and Gray, 1984) and the bean *Phaseolus angularis* (Ishiyama and Tamura, 1985; Ishiyama *et al.*, 1985).

The enzymes are usually isolated as monomeric polypeptides of 60-63 KDa. However Tamura and coworkers (Hirasawa and Tamura, 1980; Hirasawa-Soga and Tamura, 1981; Hirasawa *et al.*, 1982) reported a molecular weight of about 86 KDa for the spinach enzyme which separates into two components of 61 KDa and 24 KDa after DEAE-Sephadex A-50 chromatography. Hirasawa-Soga *et al.*, (1982) speculated that the larger component is a

modified form of the native enzyme having only methyl viologen-linked activity whilst the smaller component acts as a "coupling" protein which confers ferredoxin-linked activity. These results are at variance with the observation that purified spinach nitrite reductase of molecular weight 63 KDa can utilise ferredoxin and methyl viologen equally effectively as an electron donor (Joy and Hageman, 1966; Ida and Mikami, 1986).

The amino acid composition for the enzyme has been determined in spinach (Vega and Kamin, 1977; Ida and Mikami, 1986), *Cucurbita pepo* (Hucklesby *et al.*, 1976) and barley (Ip *et al.*, 1990) producing nearest integer values of 564, 558, 565 and 575 amino acid residues respectively. The calculated molecular weight of leaf nitrite reductase from the amino acid composition lies within the range 60-63 KDa while the spinach structural gene encodes a mature protein of molecular weight 63 KDa (Back *et al.*, 1988).

The enzyme can use purified ferredoxin (reduced with dithionite) as electron donor for *in vitro* nitrite reduction (Joy and Hageman, 1966), with ferredoxin widely accepted as the physiological electron donor in leaf tissue, reduced *in vivo* by electron transport associated with photosystem I (Neyra and Hageman, 1974). The enzyme can also utilise reduced viologen dyes (methyl and benzyl viologen) as electron donors (Hageman *et al.*, 1962; Joy and Hageman, 1966) for *in vitro* nitrite reduction, but is unable to use reduced pyridine nucleotides without the addition of the separate activity NADPH-ferredoxin reductase and ferredoxin (Hageman *et al.*, 1962).

Purified spinach leaf nitrite reductase possesses a greater affinity for nitrite with ferredoxin as electron donor, with a Michaelis constant of 10.3μM (Ida and Mikami, 1986) compared to 360μM (Ida and Morita, 1973) and 110μM (Ida and Mikami, 1986) possessed by the methyl viologen-linked activity. Serra *et al.*, (1982) reported a Km value for nitrite of 250μM obtained using either ferredoxin or methyl viologen as electron donor.

The purified enzyme has a high affinity for ferredoxin with a Km of 6μM in spinach (Ida and Mikami, 1986), while Ho and Tamura, (1973) reported a Km for ferredoxin of 70μM. Hirasawa-Soga and Tamura, (1981) reported a Km value for ferredoxin of 27μM for the 86 kDa protein species purified from spinach leaves. The enzyme from spinach expressed a Km value for methyl viologen of 64μM (Ida and Morita, 1973) compared to 120μM found by Ida and Mikami, (1986). Barley leaf nitrite reductase exhibited a Km value for methyl viologen of 120μM (Serra *et al.*, 1982).

The pH optimum of the enzyme from spinach leaves is 7.5 (Ida and Morita, 1973) but lower in barley leaf where the optimum lies within the range pH 6.0-6.5 (Serra *et al.*, 1982). The enzyme has a low thermostability (Ho and Tamura, 1973; Serra *et al.*, 1982) with enzymic activity almost completely destroyed after treatment at 60°C for 5 minutes (Ho and Tamura, 1973).

1.1.4.1. Prosthetic groups

Nitrite reductase contains sirohaem (Murphy *et al.*, 1974; Vega and Kamin, 1977) which has also been found in several sulphite reductases and shown to be an iron tetrahydroporphyrin of the isobacteriochlorin type (Murphy *et al.*, 1974). Sirohaem expresses a visible spectrum with absorption maxima in spinach nitrite reductase at 276, 386 (soret), 573 (α) and 690nm (Vega and Kamin, 1977). This characteristic spectrum has been found in purified nitrite reductase from other species including spinach (Ho and Tamura, 1973; Ida, 1977; Ida *et al.*, 1976; Hirasawa and Tamura, 1980; Ida and Mikami, 1986; Hirasawa-Soga and Tamura, 1981), *Cucurbita pepo* (Hucklesby *et al.*, 1976), bean (Ishiyama *et al.*, 1985) and barley (Serra *et al.*, 1982; Ip *et al.*, 1990).

Spectral shifts occur when the enzyme is treated with dithionite, nitrite, cyanide and carbon monoxide (Hucklesby *et al.*, 1976; Vega and Kamin, 1977) indicating some form of interaction with the haem moiety. Electron paramagnetic resonance (EPR) studies show that the untreated oxidised enzyme contains a high-spin Fe^{3+} haem (sirohaem) and that the addition of nitrite or cyanide results in a spin-state change of the haem (Aparicio *et al.*, 1975; Stoller *et al.*, 1977; Cammack *et al.*, 1978). Cyanide, a competitive inhibitor of nitrite, is assumed to bind to nitrite reductase and interact with the sirohaem (Aparicio *et al.*, 1975).

Nitrite reductase also contains an iron-sulphur centre, originally thought to be a Fe_2S_2 centre (Vega and Kamin, 1977), but shown later to be a tetranuclear Fe_4S_4 centre (Lancaster *et al.*, 1979). Haem ligands such as carbon monoxide and cyanide were found to modify both the reducibility and the EPR signal line shape of the Fe_4S_4 centre with the addition of carbon monoxide resulting in a 10-fold increase in intensity of the reduced iron-sulphur centre EPR signal (Lancaster *et al.*, 1979). This indicates an interaction between the haem and the Fe_4S_4 centre of the active site.

Photoreduction by 5'-deazaflavin and EDTA of nitrite reductase complexed with either carbon monoxide or cyanide produces shifts in the electron paramagnetic resonance

spectrum of the complex with the appearance of a g=1.94 type EPR spectrum typical of a reduced Fe_4S_4 centre (Wilkerson *et al.*, 1983). This suggests that the two prosthetic groups of nitrite reductase may interact in some way at the catalytic centre of the enzyme, with the sirohaem moiety binding nitrite and accepting electrons from the Fe_4S_4 centre which is reduced, *in vivo*, by ferredoxin or *in vitro* by methyl viologen/dithionite.

Nitrite reductase-linked sirohaem from spinach leaves possesses a midpoint potential of -50mV (Stoller *et al.*, 1977) compared to -120mV for purified nitrite reductase from *Cucurbita pepo* leaves (Cammack *et al.*, 1978) and +80mV for barley leaf nitrite reductase (Ip *et al.*, 1990). However, the midpoint potential of the iron-sulphur centre of purified nitrite reductase from spinach leaves is extremely electronegative, approximately -550mV (Stoller *et al.*, 1977) and estimated at -570mV at pH 8.1 (Cammack *et al.*, 1978) who found the midpoint potential appeared to be pH-dependent in the purified enzyme from *cucurbita pepo* leaves. Ip *et al.*,(1990) also experienced difficulty in determining the midpoint potential of such an electronegative centre in nitrite reductase from barley leaves but used a computer generated best fit procedure to determine a value of -517mV. This raised the difficulty of how ferredoxin, with a midpoint potential of -420mV could reduce the iron-sulphur centre *in vivo*. It was suggested by Stoller *et al.*, (1977) that the midpoint potential of the iron-sulphur centre, under physiological conditions, shifts to a more electropositive value, thus allowing its reduction by ferredoxin.

1.1.4.2. Location

Nitrite reductase is widely accepted to be located within the chloroplasts in higher plant leaf tissue with fractionation studies generally demonstrating the sedimentation of nitrite reductase coincidentally with chloroplast marker enzymes and chlorophyll.

Ritenour *et al.*, (1967) used a non-aqueous separation procedure with corn and foxtail leaf tissue to demonstrate localisation of nitrite reductase activity with or within the chloroplast. However, aqueous extraction and fractionation of green leaves from foxtail plants showed that 84 per cent of the total nitrite reductase activity was present in the soluble fraction. Discontinuous sucrose density gradient centrifugation showed that the distribution of nitrite reductase coincided with that of marker enzymes for whole chloroplasts (triosephosphate isomerase) in spinach and tobacco leaves (Dalling *et al.*, 1972a) and in spinach leaves (Miflin, 1974).

However, Grant *et al.*, (1970) using high rates of CO_2 fixation and O_2 evolution as a measure of chloroplast integrity found that nitrite reductase activity was present mainly in the supernatant fractions after differential centrifugation studies on spinach and sunflower. Lips and Avissar, (1972) used density gradient centrifugation to suggest that nitrite reductase was located in the peroxisomes in tobacco leaves.

The main difficulty in these studies was the fragility of the organelles which caused leakage or rupture during the isolation procedure and so allowed cross contamination between the various fractions. Adsorption and occlusion of enzymes by organelles may also produce cross contamination resulting in the conflicting reports outlined above.

Indirect evidence for the chloroplastic location of nitrite reductase has come from functional studies on isolated chloroplasts. Paneque *et al.*, (1963) demonstrated that spinach grana with added chloroplast extract could reduce nitrite in the light, while Magalhaes *et al.*, (1974) reported that intact spinach chloroplasts, capable of fixing CO_2 at high rates, reduced nitrite and synthesised amino-nitrogen *de novo* when supplied with only nitrite and light.

Nitrite reduction is functionally associated with electron transport arising from the light reactions of the chloroplast in spinach and maize (Neyra and Hageman, 1974). These workers used methyl viologen and phenazine methosulphate (photosystem I electron acceptors), 3-(3,4-dichlorophenyl)-1,1-dimethylurea (DCMU) an electron-transport inhibitor and methylamine a photophosphorylation uncoupler to show that nitrite reduction in isolated chloroplasts is associated with photosystem I, with ferredoxin the most likely physiological electron donor in leaf tissue. Purified nitrite reductase from spinach and maize would accept electrons from ferredoxin reduced either by illuminated chloroplasts or with dithionite (Joy and Hageman, 1966).

Anderson and Done, (1978), using pea, confirmed the light-dependent assimilation of nitrite by isolated chloroplasts. However, only chloroplasts isolated from plants grown in the presence of nitrate expressed nitrite reductase activity, supporting the view that the activity was due to the action of the nitrate-inducible nitrite reductase. Further support came from the observation that over short initial time periods the nitrite-dependent O_2 evolution gave oxygen to nitrite ratios that approached 1.5 reflecting the transfer of 6 mol-equivalents of electrons from 3 mole H_2O to 1 mole nitrite to form 1 mole ammonium ions (Anderson and Done, 1978; Robinson, 1986). The addition of a-ketoglutarate reduced the recovery of N as ammonium ions from 60 to 27 percent and increased the long term oxygen to nitrite ratio

(0.9-1.35), suggesting nitrite was assimilated to glutamate via the chloroplastic enzymes nitrite reductase, glutamine synthetase and glutamate synthase (Anderson and Done, 1978). Thus, the biochemical and physiological evidence suggests that nitrite reductase is located within the chloroplast in higher plants.

Since nitrite reduction is considered to occur within the chloroplasts several workers have attempted to determine whether the nitrite reductase structural gene is coded by DNA within the nucleus or within the plant organelles. Attempts have been made to infer the origin of nitrite reductase mRNA using the protein synthesis inhibitors cycloheximide and chloramphenicol which inhibit the peptidyl transferase activity of the nuclear and organellar small ribosomal subunits respectively. Cycloheximide inhibits the induction of nitrite reductase activity in *Lemna minor* when presented to the tissue before nitrate (Stewart, 1967). When cycloheximide was added after exposure to nitrate all further induction of nitrite reductase activity was prevented, suggesting that nitrite reductase was synthesised on cytoplasmic ribosomes and was coded by nuclear DNA. This view was supported by Sluiters-Scholten, (1973) who showed that the induction of nitrite reductase activity by nitrate in green leaves of *Phaseolus vulgaris* in the light was inhibited by cycloheximide. Both cycloheximide and chloramphenicol inhibited the induction of nitrite reductase activity in etiolated leaves (Sluiters-Scholten, 1973). However, chloramphenicol did not inhibit the induction of nitrite reductase activity when presented to seedlings after 24 hours illumination. Sluiters-Scholten (1973) concluded that nitrite reductase was synthesised on cytoplasmic ribosomes, and therefore presumably coded by nuclear DNA, but some form of chloroplast development is required before production of nitrite reductase molecules can occur.

Molecular studies (Small and Gray, 1984; Gupta and Beevers, 1987; Back *et al.*, 1988) suggests that nitrite reductase shares common properties with nuclear-encoded chloroplast proteins. Such proteins are usually synthesised as larger molecular weight precursors containing an amino-terminal sequence, the transit peptide (Chua and Schmidt, 1979) which is proteolytically cleaved to generate the mature functional protein during or after transport into the chloroplast (Grossman *et al.*, 1982; Robinson and Ellis, 1984; Smeekin *et al.*, 1985).

Thus, *in vitro* translation of poly A^+ RNA and immuno-precipitation of products with specific nitrite reductase antiserum reveals the synthesis of a peptide which has a higher molecular weight than the native enzyme in wheat (Small and Gray, 1984) and pea (Gupta and Beevers, 1987). In pea the *in vitro* synthesised, higher molecular weight precursor is

cleaved, in a two step process, to a peptide of the same size as that of the native enzyme by a proteinaceous extract from chloroplasts (Gupta and Beevers, 1987).

This evidence for a precursor protein has been confirmed by the molecular cloning of spinach nitrite reductase cDNA species by the use of oligonucleotide probes based on partial amino acid sequence data and through immunoscreening of a cDNA library in the expression vector lamda gt11, (Back *et al.*, 1988). These studies show that the precursor protein for nitrite reductase is 594 amino acids long (molecular weight 66,394) and has a 32 amino acid extension at the N-terminal end of the mature protein (molecular weight 62,883) which probably serves as the transit peptide.

1.1.4.3. Environmental control

Environmental control of nitrite reductase levels has not been investigated as extensively as for nitrate reductase. However, nitrite reductase activity is high in leaf tissue from plants grown on nitrate in the light (Gupta and Beevers, 1983; Small and gray, 1984; Ogawa and Ida, 1987). Nitrate is not obligatory for expression of nitrite reductase activity. Plants grown in the light in the absence of nitrate possess low, but measurable levels of leaf nitrite reductase activity (about 10-30 per cent of nitrate-grown plants) (Gupta and Beevers, 1983; Ogawa and Ida, 1987).

The level of nitrite reductase activity in leaf tissue is also influenced by light. Plants exposed to nitrate in the dark possess low levels of leaf nitrite reductase activity (about 30-50 per cent of light-grown, nitrate-treated plants) (Gupta and Beevers, 1983; Ogawa and Ida, 1987).

Immunological studies (Gupta and Beevers, 1984) suggests that regulation of nitrite reductase activity levels by nitrate and light are due to the *de novo* synthesis of nitrite reductase molecules. Experiments with spinach nitrite reductase cDNA as a hybridisation probe shows that nitrate application leads to a large increase in the steady-state level of nitrite reductase apoprotein mRNA (Back *et al.*, 1988). Whether this is due to modulation of transcription or to stabilisation of the mRNA transcripts remains to be resolved. Environmental control of nitrite reductase is discussed more fully in chapter 6.

1.1.5. Nitrite reductase in non-chlorophyllous tissue

Nitrite reductase activity has been found in non-chlorophyllous tissues such as maize scutellum, where two isozymes were identified (Hucklesby *et al.*, 1972; Dalling *et al.*, 1973) with similar properties to each other and leaf nitrite reductase.

Nitrite reductase activity also occurs in root tissue and is believed to be located within root plastids (Dalling *et al.*, 1972; Miflin, 1974; Emes and Fowler, 1979). The enzyme is similar to leaf nitrite reductase, utilising dithionite-reduced viologen dye as reductant in *in vitro* assays (Sanderson and Cocking, 1964b). Nitrite reductase activity is increased in plastids isolated from root tissue from nitrate-grown pea which also possess an increased flow of carbon through the pentose phosphate pathway compared to root plastids from peas grown in the absence of nitrate (Emes and Fowler, 1983). Plastids from root tissue from nitrate-grown barley plants also contain a glucose-6-phosphate and $NADP^+$-linked nitrite reductase system (Oji *et al.*, 1985) with both benzyl and methyl viologen enzymatically reduced by plastid extract in the presence of glucose-6-phosphate and $NADP^+$. The identification of a pyridine nucleotide reductase immunologically similar to spinach leaf ferredoxin-$NADP^+$ reductase (Suzuki *et al.*, 1985), and of a ferredoxin-like electron carrier (Ninomiya and Sato, 1984; Suzuki *et al.*, 1985), support the suggestion that the supply of reducing power for *in vivo* root nitrite reduction originates from the pentose phosphate pathway.

Purified nitrite reductase from pea roots has a molecular weight of about 60 KDa and exhibits absorption maxima at 278, 384, 573 and 695nm (Bowsher *et al.*, 1988) similar to spinach leaf nitrite reductase (Vega and Kamin, 1977). The electron paramagnetic resonance spectrum is indistinguishable from that of the *Cucurbita pepo* leaf enzyme (Bowsher *et al.*, 1988), while reduction of the purified enzyme with dithionite in the presence of cyanide allows the appearance of a signal around g=1.94, characteristic of an iron-sulphur centre (Bowsher *et al.*, 1988).

The purified protein from pea root reacts with antibodies raised against *Cucurbita pepo* leaf nitrite reductase in Ouchterlony double diffusion experiments (Bowsher *et al.*, 1988). Nitrite reductase from leaf and root tissues produce single lines of identity in pea (Bowsher *et al.*, 1988) and bean (Ishiyama *et al.*, 1985) in Ouchterlony double diffusions suggesting that, in these species at least, nitrite reductase is very similar in both leaf and root tissues.

1.2. Molecular analysis of nitrate assimilation

1.2.1. Nitrate reductase

Complementary DNA encoding nitrate reductase has been isolated in barley (Cheng *et al.*, 1986) squash (Crawford *et al.*, 1986) and tobaco (Calza *et al.*, 1987). These cDNA clones have been used as probes to isolate the nitrate reductase structural gene and for Northern blot analysis to study the regulation of nitrate reductase (Caboche *et al.*, 1989; Crawford and Davis, 1989) and in the characterisation of nitrate reductase-deficient mutants (Kleinhofs *et al.*, 1989).

1.2.2. Nitrite reductase

Complementary DNA encoding nitrite reductase has been isolated from spinach (Back *et al.*, 1988) and maize (Lahners *et al.*, 1988). The cloned nitrite reductase cDNA from spinach codes for a precursor protein that contains 594 amino acids with a deduced molecular weight of 66,394 (Back *et al.*, 1988). The nitrite reductase precursor protein is 32 amino acids longer than the mature protein which contains 562 amino acids and has a deduced molecular weight of 62,883 (Back *et al.*, 1988). This value corresponds well with the apparent molecular weight of 61kDa (Ho and Tamura, 1973; Vega and Kamin, 1977). The additional amino acids precede the mature protein and are likely to act as a transit peptide sequence in directing the nuclear-encoded nitrite reductase protein into the chloroplast (Back *et al.*, 1988).

The amino acid sequence deduced from the spinach cDNA sequence contains 4 cysteine residues in positions 473, 479, 514 and 518 (Back *et al.*, 1988) that are probably involved in the binding of the tetranuclear iron cluster (Aparicio *et al.*, 1975; Lancaster *et al.*, 1979). These cysteines are conserved in the maize cDNA sequence in exactly the same location in the protein (Lahners *et al.*, 1988).

The spinach nitrite reductase cDNA has been used as a probe to isolate a genomic clone from spinach DNA. The coding region in the nitrite reductase gene contains 3 introns (Back *et al.*, 1989). Southern blot analysis has confirmed that, in spinach, a single nitrite reductase gene is present per haploid genome (Back *et al.*, 1989). However, there is evidence to suggest the presence of 2 nitrite reductase genes per haploid genome in maize (Lahners *et al.*, 1988). This correlates with the presence of two nitrite reductase isozymes in maize (Dalling *et al.*, 1973; Kutscherra *et al.*, 1987).

Nitrate induction of nitrite reductase activity due to *de novo* synthesis of enzyme molecules has been well documented for several plant species (Beevers and Hageman, 1969; Gupta and Beevers, 1984, 1987; Small and Gray, 1984, Ogawa and Ida, 1987). Northern blot analysis has shown that nitrite reductase mRNA increases considerably in spinach leaves (Back *et al.*, 1988) and maize leaf and root tissue (Lahners *et al.*, 1988) from plants treated with nitrate.

There is a rapid induction of nitrite reductase mRNA after the addition of nitrate in maize with significant increases in the level of nitrite reductase mRNA in root tissue after 30 minutes and in leaf tissue after 90 minutes (Kramer *et al.*, 1989). This timing difference may reflect the time at which inducing concentrations of nitrate become available to the different organs.

The stability of nitrite reductase mRNA is very low in maize, having a half-life of less than 30 minutes in root and 40 minutes in leaf tissue (Kramer *et al.*, 1989). Thus the level of nitrite reductase mRNA decreased rapidly after a maximum RNA level was reached (Kramer *et al.*, 1989) even though the plants were still exposed to nitrate.

Thus, the increase in nitrite reductase activity in response to nitrate due to *de novo* synthesis of enzyme molecules is preceded by a transient increase in the steady-state levels of nitrite reductase mRNA. Whether the increase in the level of nitrite reductase mRNA is due to changes in transcription rate, altered RNA processing or decreased mRNA degradation remains to be determined.

1.3. Nitrate assimilation mutants

1.3.1. Mutant analysis

The isolation and characterisation of mutant alleles which define genetic loci is a powerful tool in the study of metabolism as evidenced by its classical and continued application to microbial systems and, more recently, its application to higher plants (reviewed in Blonstein and King, 1986). Mutant analysis allows not only the identification of genetic loci whose existence can be deduced *a priori* from a knowledge of the biochemical characteristics of the pathway but also it should allow the identification of genes which encode previously unknown components of the pathway. Of special interest among these being genes whose products contribute to the regulatory networks controlling metabolism. Thus, mutant analysis may lead to the identification (and subsequent manipulation) of single genes of agronomic importance allowing more efficient crop production.

1.3.2. Regulation

There have been no reports of regulatory mutations affecting nitrate assimilation in higher plants so far. In *Nicotiana tabacum* two *cnx* A alleles (Cnx 68 and Cnx 101) produce constitutive levels of nitrate reductase apoprotein and also show an increased constitutive nitrite reductase, as do the *nia* allele Nia 102 and *cnx* B alleles (Mendel and Muller, 1979; Mendel *et al.*, 1984; Schiemann and Muller, 1985). Whether the constitutive levels of nitrate reductase and nitrite reductase activity are a consequence of the MoCo or apoprotein gene mutation is not clear. However in *Aspergillus nidulans* some mutations causing loss of a functional nitrate reductase molecule lead to constitutive synthesis of both nitrate and nitrite reductase and suggest an autoregulatory role for functional nitrate reductase molecules in the regulation of synthesis of both nitrate reductase and nitrite reductase (Pateman *et al.*, 1967; Cove and Pateman, 1969).

There is, however, little other evidence in the literature (Wray, 1986) for an autoregulatory role for nitrate reductase in higher plants and there is no *a priori* reason to suppose that the regulatory networks operating in higher plants are the same as those in lower eukaryotes. Indeed, the involvement of light in the regulation of the higher plant enzymes suggests that they are different, at least in part.

1.3.3. Nitrate uptake

Nitrate uptake mutants are poorly represented amongst the reports of nitrate assimilation mutants so far identified (Doddema and Telkamp, 1977; Wallsgrove, 1987). The lack of a positive selection scheme for the identification of nitrate uptake mutants forces the adoption of total isolation procedures (Wallsgrove, 1987) which are both labour intensive and time consuming.

1.3.4. Nitrate reduction

A great number of mutants altered in nitrate reduction have been isolated in higher plants. These mutants carry mutations either in the nitrate reductase apoprotein structural genes or in genes involved in molybdenum cofactor synthesis and insertion (reviewed in Dunn-Coleman *et al.*, 1984; Kleinhofs *et al.*, 1985; Wray, 1986; Wray 1988). That such a large number of mutants altered in nitrate reduction have been isolated is undoubtedly due to the availability of chlorate as a positive selection agent (Oostindier-Braaksma and Feenstra, 1972; Braaksma and Feenstra, 1982a,b) allowing the identification of plants lacking a functional nitrate reductase activity by a simple visual screening procedure.

1.3.5. Nitrite reduction

The only plant variants defective in nitrite reduction were described in *Haplopappus gracilis* (Gilissen *et al.*, 1985). They selected a callus (Ao) which was able to grow in the presence of normally toxic levels of asparagine. After X-irradiation this line was plated on medium containing alanine as nitrogen source and calli growing at wild type rates were selected. Two lines failed to grow on medium containing nitrate as a nitrogen source and excreted nitrite. While *in vitro* nitrate reductase activity was similar to wild type, both *in vivo* nitrate reductase and *in vitro* nitrite reductase activities were only 20-40 per cent of the wild type level. Attempts at plant regeneration were unsuccessful and the molecular basis of the defects are unknown.

Whole plant mutants defective in nitrite reduction are of particular significance since they would allow not only the characterisation of the number and type of genes which determine this step in nitrate assimilation, but also the consequences of such mutations in the intact differentiated organism. A further consideration is that some types of mutation may only be expressed, and thus may only be selected, at the whole plant level. However, as

yet, there have been no reports in the literature of whole plant mutants defective in nitrite reduction.

Whole plants carrying a homozygous mutation responsible for defects in nitrite reduction should be identifiable within M_2 populations of barley (treated with sodium azide in the M_1 generation) by virtue of the fact that the defect might be expected to cause an accumulation of nitrite (Dunn-Coleman *et al.*, 1984; Wray, 1986) within plant tissues after treatment with nitrate.

1.4. Aims

The aims of the work described here were:

i. To isolate mutant barley plants defective in nitrite reduction by identifying individuals which accumulate nitrite after treatment with nitrate.

ii. To biochemically characterise selected plants to determine the basis for nitrite accumulation.

iii. To genetically characterise selected plants by allelism tests through cross pollinations between selected nitrite accumulators and the identification, by subsequent progeny segregation, of heterozygous lines carried on selected M_1 spikes, or produced by cross pollination between selected nitrite accumulators and wild type plants.

iv. To develop a fuller understanding of the biochemistry and physiology of nitrite reduction in wild type barley plants which may be used in the characterisation of selected nitrite accumulators.

Chapter 2

Materials and methods

Materials

2.1.1. Chemicals

All biochemicals and common chemicals used were of standard laboratory grade unless otherwise stated. Where appropriate, chemical formulae and common names used in the text are given.

5-bromo-4-chloro-3-indolyl phosphate, disodium salt (BCIP), Coomassie brilliant blue G-250, cysteine HCl, 1,1'-dimethyl-4-4'-bipyridinium chloride (methyl viologen), diphenyl-3,3`-(3,3`-dimethoxy-4,4`-diphenylene) ditetrazolium chloride grade III, crystalline (nitroblue tetrazolium, NBT), flavin adenine dinucleotide disodium salt, (FAD), gentamicin sulphate, glutamine, glycine, goat antirabbit alkaline phosphatase conjugate, molecular weight markers (prestained kit MW-SDS-BLUE 27,000-180,000) for SDS gel electrophoresis (triosephosphate isomerase (rabbit muscle), 26,600 Da, lactic dehydrogenase (rabbit muscle), 36,500 Da, fumarase (porcine heart), 48,500 Da, pyruvate kinase (chicken muscle), 58,000 Da, fructose-6-phosphate kinase (rabbit muscle), 84,000 Da, β-galactosidase (E.Coli), 116,000 Da, α_2-macroglobulin (human plasma), 180,000 Da), β-nicotinamide adenine dinucleotide, reduced form, disodium salt from yeast, grade III, (β-NADH), 2[N-morpholino] ethanesulphonic acid (MES), triphenyl tetrazolium chloride were obtained from Sigma Chemical Co. Ltd. Poole, Dorset, England.

Acrylamide, grade I "Electran", ammonium persulphate, calcium chloride ($CaCl_2.6H_2O$), chloroform, copper sulphate ($CuSO_4.5H_2O$), ethylenediaminetetra-acetic acid, disodium salt (EDTA), ferric monosodium salt (NaFe EDTA), ethanol, glycerol, hydrochloric acid "AristaR" grade, magnesium chloride ($MgCl_2.6H_2O$), manganese sulphate ($MnSO_4.5H_2O$), methanol, N N'-methylene bisacrylamide, electrophoresis grade, β-mercaptoethanol, N-1-naphthylethylenediamine dihydrochloride (NED), N,N,N`,N`-tetramethyl-ethylene diamine ethane (TEMED), orthophosphoric acid "AristaR" grade, potassium chloride, potassium phosphate (KH_2PO_4/K_2HPO_4), potassium nitrate, potassium nitrite, sodium azide (NaN_3), sodium dithionite ($Na_2S_2O_4$), sodium phosphate (NaH_2PO_4/Na_2HPO_4), sucrose "AnalaR" grade, sulphanilamide, sulphuric acid "AristaR" grade, trisodium citrate were obtained from British Drug Houses Ltd, Poole, Dorset, England.

Formvar powder (polyvinyl formal), glutaraldehyde (EM grade), lead nitrate, L.R. White resin (medium grade), nickel specimen grids (100 mesh hexagon, 340μm opening), osmium tetroxide, paraformaldehyde (EM grade), Spurr resin kit, uranyl acetate were obtained from Agar Scientific, Stansted, Essex, England.

Goat anti-rabbit IgG-gold conjugate (15nm, GAR G15) was obtained from Janssen Life Sciences Products, Wantage, Oxon, England.

Ammonium molybdate ($(NH_4)_6Mo_7O_{24}.4H_2O$), bromophenol blue, sodium chloride, sodium molybdate ($Na_2Mo_4O_4.2H_2O$), were obtained from Fisons Ltd, Loughborough, Leicestershire, England. Boric acid, magnesium sulphate ($MgSO_4.7H_2O$), sodium bicarbonate were obtained from May and Baker Ltd, Dagenham, England. Tris(hydroxymethyl)aminomethane (Tris), and bovine serum albumin, fraction V, (BSA) were obtained from Boehringer Mannheim, GmbH, West Germany. 2-chloro-6-(trichloromethyl)pyridine (N-SERVE®) was obtained from Dow Chemical Co. Kings Lynn, Norfolk, England. Diphenylamine sulphonic acid chromogene (Szechrome NAS®) was obtained from Polysciences Inc. Warrington, England. Marvel (milk powder, Cadbury Schweppes Ltd, Bournville, Birmingham, England) was obtained from a local supermarket. Nitrocellulose filters (0.45μm) were obtained from Schleicher and Schuell, Dassel, West Germany and sodium dodecyl sulphate (SDS), electrophoresis grade was obtained from National Diagnostics, Aylesbury, Bucks, England.

2.1.2. Seed and plant growth materials

Barley seed was obtained from William Watt, Seed Merchants, Cupar, Scotland and the Scottish Crop Research Institute, Invergowrie, courtesy of Dr W. Thomas.

Bulk-harvested M_2 barley seed, cv. Golden Promise, Klaxon, Patty, Apex, Maris Mink (azide-treated in the M_1) was obtained from Rothamsted Experimental Station, Harpenden, Herts, courtesy of Dr R. Wallsgrove during the period 1980-1985.

Spike-harvested M_2 barley seed (cv. Corniche and Digger, 1987) was produced by mutagenesis with sodium azide in the M_1 generation and grown in field plots at Rothamsted Experimental Station, Harpenden, Herts., courtesy of Dr R. Wallsgrove.

Spike-harvested M_2 seed cv. Golden Promise, Klaxon, Tweed, Natasha, Vista, Doublet, 1987 (azide-treated in the M_1) was grown at the Scottish Crop Research Institute, Invergowrie, courtesy of Dr. W. Thomas.

Vermiculite was obtained from Hoben Davis Ltd, Newcastle, Staffs, England. Potting compost (Humax®) was obtained from the local botanic gardens.

Methods

2.2. Mutant production, isolation and maintenance

2.2.1. Seed mutagenesis

Wild type seeds of barley (M_1 seed) were mutagenised by chemical mutagenesis using the procedure described by Kleinhofs *et al.*, (1978). Seed of various cultivars were hydrated in trays of tap water at 0-4°C for 16 hours and then transferred to 10 litre flasks containing 3-4 litres of tap water which was aerated vigorously for a further 8 hours at room temperature. The flasks were placed in a fume cabinet and the water was replaced with 3 litres 0.1M potassium phosphate buffer pH 3 and sodium azide solution added to the buffer to give a final concentration of 1mM. After aeration of this solution for a further 2 hours, the seeds were washed in several changes of tap water for 30 minutes, blotted to remove excess liquid and redried.

2.2.2. Growth of M_1 plants and collection of M_2 seed

Barley seed mutagenised courtesy of Dr.R.Wallsgrove as described above was grown in field plots at Rothamsted Experimental Station, Harpenden, Herts. Seed mutagenised as described above was also grown in field plots at the Scottish Crop Research Institute, Invergowrie, courtesy of Dr.W.Thomas.

Bulk-harvested single M_2 seed was collected with a combine harvester, or individual M_1 spikes carrying M_2 seed were collected manually. 60,000 spikes were dried and then individually bagged and numbered.

2.2.3. Screening for *in vivo* nitrite accumulators

2.2.3.1. Bulk-harvested populations

Bulk-harvested M_2 seed was sown in trays of vermiculite to give a germination rate of about 200 seedlings per tray, and watered with nitrate-less half-strength Hoaglands nutrient solution (0.2mM NaFe EDTA, 0.5mM KH_2PO_4, 1mM $MgSO_4.7H_2O$, 0.4μM $ZnSO_4.7H_2O$, 0.2μM $MnSO_4.5H_2O$, 0.02mM H_3BO_3, 0.05μM $Na_2Mo_4O_4.2H_2O$; Hoagland and Arnon, 1938) in the dark. After 4 days the germinated seedlings were transferred into the light ($115\mu Em^{-2}s^{-1}$), and after a further 2 days growth the seedlings were watered with half-strength Hoaglands nutrient solution containing 25mM KNO_3 and maintained in the

Fig 2.1. Identification of nitrite accumulating individuals within a nitrite positive section.

Seedlings within a section producing a positive nitrite reaction were individually tested to determine which seedling, or seedlings, were responsible for the positive reaction. To ensure each individual within the section was sampled, and to aid identification of the seedling, or seedlings, responsible for the positive nitrite reaction, numbered markers were placed beside each seedling as tissue was removed for analysis of nitrite accumulation.

Fig 2.2. Sowing of M_2 seed carried on M_1 spikes.

M_2 seed from individually bagged and numbered M_1 spikes were sown such that up to 5 seed from each spike were placed together in a unique position in a numbered grid placed over the vermiculite. Once every position in the grid was filled the seed was covered with a light sprinkling of vermiculite so that the seeds were covered yet the grid was still visible. After germination, the 7 day old seedlings were screened for nitrite accumulation.

light overnight. The following day the 7 day old seedlings were screened for defects in nitrite reduction by testing leaf tissue for accumulation of nitrite.

Seedlings were screened for nitrite accumulation by firstly dividing each tray into sections of about 50 plants with wooden markers. The leaf tips (about 10mm in length) from each plant within the section were pooled and then homogenised by grinding the tissue in a mortar and pestle in 1ml H_2O, 1ml 1% sulphanilamide in 3N HCl and 1ml 0.02% NED. The homogenate was centrifuged for 5 minutes at 1,200g in a bench centrifuge. Nitrite present in the supernatant, due to *in vivo* nitrite accumulation, reacts with the acidified sulphanilamide and NED in a diazo-coupling reaction (Snell and Snell, 1949), to form an azo dye with a characteristic pink colour.

When pools of leaf tissue producing a positive nitrite reaction were identified all plants within the pool were individually rescreened for nitrite accumulation. In the process of initial section screening it was possible to determine which plants had already been sampled since the top 10mm of the primary leaf had been cut off, while those yet to be sampled remained intact. However, during individual rescreening, all plants lacked the top 10mm of the primary leaf. Therefore, a numbered marker was placed beside each plant as it was separately rescreened to ensure every plant within the section was rescreened and to facilitate the identification of the plant or plants responsible for the positive nitrite reaction (Fig 2.1).

2.2.3.2. Spike-harvested populations

Spike-harvested M_2 seed was sown in grids placed in trays of vermiculite such that up to 5 seed from each spike were sown together in a unique position in the numbered grid (Fig 2.2). Once all the grid positions were full the seed was lightly covered with vermiculite so that the grid was still visible. The trays were watered with nitrate-less half-strength Hoaglands nutrient solution and left in the dark. After 4 days the germinated seedlings were transferred into the light ($115\mu Em^{-2}s^{-1}$) for 2 days. The green seedlings were then watered with half-strength Hoaglands nutrient solution containing 25mM KNO_3 and maintained in the light overnight. On the following day the 7 day old seedlings were screened for *in vivo* nitrite accumulation.

Seedlings were screened for nitrite accumulation by firstly dividing each tray into 4 sections. The leaf tips (about 10mm in length) from each plant within the section were then pooled and homogenised by grinding the tissue in a mortar and pestle in 1ml H_2O, 1ml 1%

sulphanilamide in 3N HCl and 1ml 0.02% NED. The homogenate was centrifuged for 5 minutes at 1,200g in a bench centrifuge. Nitrite present in the supernatant, due to *in vivo* nitrite accumulation, reacts with the acidified sulphanilamide and NED in a diazo-coupling reaction (Snell and Snell, 1949), to form an azo dye with a characteristic pink colour.

When pools of leaf tissue producing a positive nitrite reaction were identified leaf tissue from the plants within each separate grid position from the section were pooled and rescreened for nitrite accumulation. Thus, it was possible to identify which grid position the nitrite accumulating plant, or plants, occupied and so from which spike the nitrite accumulating plant, or plants, were derived. Each plant within the grid position responsible for the positive nitrite reaction was then individually rescreened to identify the individual plant, or plants, responsible for the nitrite accumulation.

2.2.4. Screening for *in vitro* nitrite accumulators

Individual seed was sown in unique positions in a numbered grid placed in a tray of vermiculite then given a light covering of vermiculite so that the grid was still visible. The seedlings were germinated in the dark for 4 days then transferred to the light ($115\mu Em^{-2}s^{-1}$) for 2 days growth and watered with nitrate-less half-strength Hoaglands nutrient solution.

The leaf tips (about 10mm in length) from each plant were placed in separate test tubes to which 1ml 0.1M KNO_3 was added. The tubes were then placed in the light ($115\mu Em^{-2}s^{-1}$) for 16-20 hours. The following day the solution is decanted into clean test tubes to which 1ml 1% sulphanilamide in 3N HCl and 1ml 0.02% NED is added. Nitrite accumulation is identified by the production of an azo dye with a characteristic pink colour (Snell and Snell, 1949). It was then possible to identify the plant or plants responsible for the nitrite accumulation.

2.2.5. Growth of nitrite accumulating plants

Plants identified as nitrite accumulators were rescued from the nitrate-treated vermiculite and grown in the environmentally controlled hydroponic system described by Bright *et al.*, (1983). The plants were grown in continuous light at a temperature of $20^{\circ}C$ with constant aeration of the hydroponic medium (1.3mM KH_2PO_4, 0.1mM NaFe EDTA, 1.5mM $MgSO_4.7H_2O$, 1.2mM $CaCl_2.6H_2O$, 0.1mM H_3BO_3, 0.5μM $(NH_4)_6Mo_7O_{24}.4H_2O$, 18μM $MnSO_4.5H_2O$, 2μM $ZnSO_4.7H_2O$, 2.4μM $CuSO_4.5H_2O$, 0.09mM KCl, 4μl/litre H_2SO_4, 5mM MES pH 6.0, 1mM glutamine).

To avoid the build up of microbial contamination the solution was changed every second day and the plant roots thoroughly rinsed with distilled water. Before reuse pots were rigorously washed with concentrated HCl and thoroughly rinsed with distilled water. Gentamicin (10mg/litre) was added to the hydroponic medium whenever the roots exhibited signs of microbial growth.

2.3. Genetic analysis

2.3.1. Cross pollination

Plants identified as nitrite accumulators, and which had been maintained to flowering in the hydroponic system, were reciprocally cross pollinated with wild type plants by hand. The plant destined to be the female parent (pollen recipient plant) was emasculated when the tiller had swollen and the awns on the spike appeared up to one inch from the top of the flag leaf. Spikes were emasculated by cutting open every seed sac carried on the spike and plucking out the anthers (3 anthers per seed sac), while still green, with fine forceps. The spike was left for 2-3 days covered by a glassine bag to prevent uncontrolled cross pollination from other plants. If self pollination had inadvertantly occurred, fertilisation and initiation of seed formation was visible after 2-3 days. In these cases the spike was discarded. Once the seed sacs were gaping and the ovaries ready to accept pollen, as indicated by their white fluffy appearance, anthers were individually removed, with fine forceps, from the plant destined to be the male parent (pollen donor plant) and gently tapped over the ovaries and then left in the seed sac. A spike is ready for pollen donation when the anthers (yellow and swollen in appearance) protrude from the seed sac within 10-15 minutes of the sac being cut open. Once pollination of the whole spike was complete, it was again covered with the glassine bag and the spike left to produce seed.

2.3.2. M_1 spike analysis

The remaining seed from M_1 spikes identified as carrying nitrite accumulating seedlings were germinated and screened as described (section 2.2.3.). Any seedlings expressing the nitrite accumulation phenotype were transferred to the hydroponic growth system (section 2.2.5.) while those seedlings expressing the wild type phenotype of non-accumulation were grown on in compost, in the greenhouse. These plants were allowed to self pollinate and the M_3 generation from each individual M_2 plant analysed for segregation of the nitrite accumulating phenotype.

2.4. Biochemical analysis

2.4.1. Plant growth

2.4.1.1. Wild type plants

Wild type barley seeds used in the study of the regulation of nitrite reductase under varying conditions of light and nitrogen nutrition were sown in trays of vermiculite that had been thoroughly washed in several changes of distilled water until the excess rinsing solution gave a negative nitrite reaction. The seeds were germinated in the dark at 25°C for 4 days, after which they were placed in the light (115 $\mu Em^{-2}s^{-1}$) for 2 days and watered with nitrate-less half-strength Hoaglands nutrient solution by which time the leaves were fully expanded and green. The plants were then exposed to various growth conditions with light, dark and nitrogen-containing compounds.

2.4.1.2. M_3 progeny derived from M_2 selections

M_3 progeny seeds from M_2 nitrite accumulating selections were sown individually in small pots or in unique positions within a grid placed in trays of washed vermiculite. The seeds were germinated in the dark at 25°C for 4 days, and watered with half-strength Hoaglands nutrient solution without nitrate. The seedlings were then transferred into the light (115$\mu Em^{-2}s^{-1}$) for 2 days by which time the leaves were fully expanded and green. The plants were then watered with half-strength Hoaglands nutrient solution containing 25mM KNO_3 and after a further 24 hours in the light the plants were harvested for analyses.

2.4.2. Analysis of tissue from hydroponically-maintained M_2 plants

Analyses of M_2 nitrite accumulating plants at or shortly after selection proved difficult by conventional extraction and enzyme assay methods, since leaf tissue had already been removed during screening and the amount of tissue remaining for analysis was small. Removal of tissue appeared to impair the growth and survival of the plant, so that no more than about 10mg tissue could be removed for analysis after the plant had been exposed to nitrate. However, once the plant was established on the hydroponic growth system, with glutamine as nitrogen source, tissue could be removed and analysed more frequently. However, before analysis for nitrate and nitrite reductase activities the glutamine grown tissue had to be induced. Since it was not practicable to expose the whole plant to nitrate, leaf sections were removed and vacuum infiltrated with nitrate as described below.

Tissue (about 10mg) was floated on 30ml 0.1M KNO_3 in separate 100ml conical flasks which were and placed in a dessicator. A vacuum was applied until air bubbles were forced from the tissue and the solution began to bubble. This was repeated twice more. The flasks containing the vacuum infiltrated tissue and 0.1M KNO_3 solution were placed in the light ($115\mu Em^{-2}s^{-1}$) overnight.

Some 16-18 hours later tissue was analysed by western blotting, nitrate reductase and nitrite reductase enzyme assays.

2.4.3. Tissue extraction

Plant tissue (leaf or root) was extracted in 50mM Tris buffer pH 7.5, 10 mM EDTA, 10mM β-mercaptoethanol and 10% glycerol (NiR extraction buffer (Ip *et al.*, 1990)) by grinding in a mortar and pestle. Tissue to buffer ratios varied between 1:3 to 1:10 (wt:vol) depending on the amount of tissue available for analysis. The homogenate was centrifuged at 30,000g in a MSE High Speed Super 18 centrifuge for 15 minutes and the supernatant collected as the enzyme source. All procedures were carried out at $4^{o}C$.

2.4.3.1. Tissue micro-extraction

Analysis of single plants exposed to nitrate by either normal growth or tissue vacuum infiltration required the extraction of amounts of tissue too small for normal methods. Consequently, tissue, normally about 10mg, was homogenised in Eppendorf tubes with extraction buffer, with a finger held homogeniser, at various weight:volume ratios, usually 1:5 or 1:10, depending on the amount of tissue. The Eppendorf tubes were microfuged at 10,000 rpm in a bench microfuge for 10 minutes, and the supernatant used for analyses.

2.4.4. Enzyme assays

2.4.4.1. *In vitro* NADH-nitrate reductase

In vitro NADH-nitrate reductase activity was assayed according to the method of Wray and Filner, (1970). The assay mix contained 50mM potassium phosphate buffer, pH 7.5, 10mM KNO_3, 0.1mM NADH, and enzyme extract (usually 0.1ml) in a final volume of 1ml. The assay, initiated by the addition of the enzyme extract, was performed at 25^{0}C for 20 minutes, and terminated by the addition of 1ml 1% sulphanilamide in 3N HCl followed by 1ml 0.02% NED. Full colour development occurred within 15 minutes after which the tubes were centrifuged at 1,200g in a bench centrifuge for 4 minutes and the optical density of the supernatant read at 540nm (Snell and Snell, 1949). The amount of nitrite produced was calculated from a previously established standard curve (0-100 nmoles KNO_2, (Fig 2.3)). Controls were performed in the same way as above except the enzyme extract was withheld until after addition of the sulphanilamide in 3N HCl.

2.4.4.2. *In vitro* methyl viologen nitrate reductase

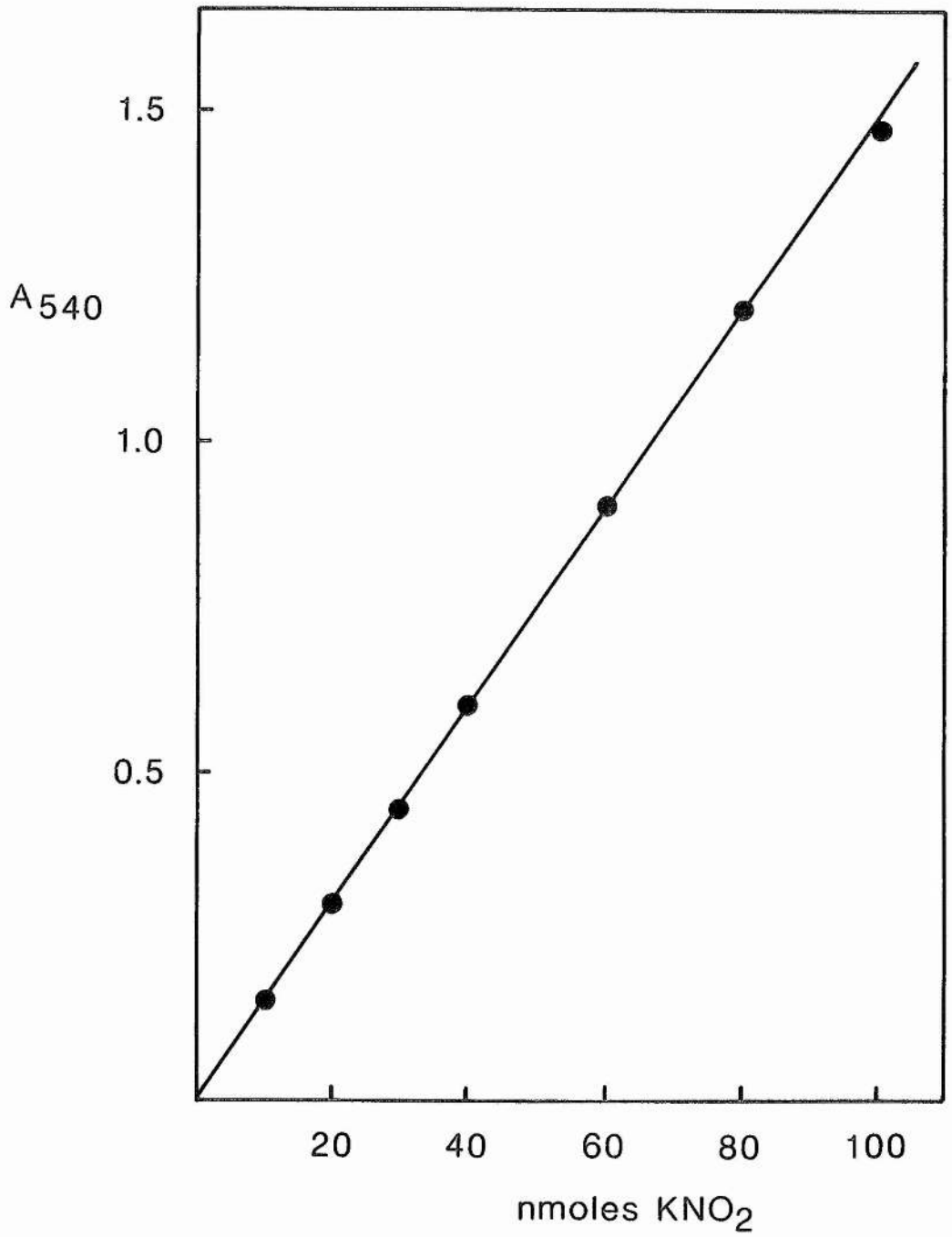
The *in vitro* methyl viologen nitrate reductase assay mix consisted of 50mM potassium phosphate pH 7.5, 10mM KNO_3, 1mM methyl viologen and 0.1ml enzyme extract in a final volume of 1ml. The assay was started by the addition of 0.2ml 10mg/ml sodium dithionite in 95mM sodium bicarbonate. After incubation at 25^{0}C for 20 minutes the reaction was stopped by vigorous aeration of the assay mix which oxidised the blue, reduced methyl viologen to the colourless leuco form. 1ml 1% sulphanilamide in 3N HCl and 1ml 0.02% NED were added and the tubes allowed to stand for 15 minutes for full colour development. The tubes were centrifuged at 1,200g in a bench centrifuge for 4 minutes and the optical density of the supernatant read at 540nm (Snell and Snell, 1949). The amount of nitrite produced was calculated from a previously established standard curve (0-100 nmoles KNO_2, (Fig 2.3)). Controls were performed by withholding the enzyme extract until after the addition of the sulphanilamide in 3N HCl.

2.4.4.3. Total-extract NADH-nitrate reductase

Nitrate reductase activity was assayed in a total-extract assay using the method of Wray *et.al.*, (1985). Small pieces of tissue (5-10mg) were ground in a mortar with 1ml of 50mM potassium phosphate buffer, pH 7.5, containing 0.1mM EDTA, 10μM FAD, and 1mM cysteine. The brei was transferred to a test-tube, 0.1ml of 0.1M KNO_3 and 0.1ml of 1mM

Fig 2.3. Nitrite standard curve.

The plot shows the direct relationship between standard amounts of nitrite and optical density at 540nm after the addition of sulphanilamide and NED, over the range 0-100nmoles KNO_2.


1.5
1.0
0.5
A_{540}
20
40
60
80
100
nmoles KNO_2

NADH were added and after incubation at 25°C for 30 minutes, the reaction was terminated by the addition of 1ml of 1% sulphanilamide in 3N HCl and 1ml 0.02% NED. Full colour development occurred within 15 minutes after which the tubes were centrifuged at 1,200g in a bench centrifuge for 4 minutes and the optical density of the supernatant read at 540nm (Snell and Snell, 1949). The amount of nitrite produced was determined from a previously established standard curve (0-100 nmoles KNO_2, (Fig 2.3)). Controls were performed by withholding the NADH until after the addition of the acidified sulphanilamide.

2.4.4.4. *In vivo* nitrate reductase

Leaf tissue (about 10mg) was transversely sliced into 1mm strips with a scalpel and placed in test tubes containing 1ml assay mix (0.1M potassium phosphate buffer pH 7.5, 2% propanol, 10mM KNO_3). The tubes were placed in a dessicator and a vacuum applied until air bubbles were forced from the leaf tissue and the assay mix began to bubble. This procedure was repeated twice after which the tissue no longer floated on the surface of the solution. The tubes were then placed in the dark for 1 hour and the assay stopped by the addition of 1ml 1% sulphanilamide in 3N HCl plus 1ml 0.02% NED. Full colour development occurred within 15 minutes after which the tubes were centrifuged at 1,200g for 4 minutes in a bench centrifuge and the optical density of the supernatant read at 540nm (Snell and Snell, 1949). The amount of nitrite produced was determined from a previously established calibration curve (0-100 nmoles KNO_2, (Fig 2.3)).

2.4.4.5. *In vitro* methyl viologen nitrite reductase

In vitro nitrite reductase activity was assayed according to the method of Wray and Filner, (1970) except the tubes were not flushed with nitrogen.The assay mix contained 50mM potassium phosphate pH 7.5, 2mM KNO_2, 1mM methyl viologen, 0.1ml enzyme extract in a final volume of 1ml. The reaction was started by the addition of 0.2ml 10mg/ml sodium dithionite in 95mM sodium bicarbonate. Controls consisted of the above but lacked sodium dithionite. The assays were carried out at 25°C for 20 minutes and were terminated by vigorous aeration of the assay mix which oxidised the blue reduced methyl viologen to the colourless leuco form. 30µl of the assay mix was added to 970µl H_2O followed by 1ml 1% sulphanilamide in 3N HCl and 1ml 0.02% NED. Full colour development occurred within 15 minutes after which the optical density was read at 540nm (Snell and Snell, 1949). The amount of nitrite reduced was calculated from a previously established standard curve (0-100 nmoles KNO_2, (Fig 2.3)).

2.4.4.6. Total-extract methyl viologen nitrite reductase

Small pieces of tissue (5-10mg) were ground in a mortar with 0.8ml of 50mM potassium phosphate buffer pH 7.5, 2mM KNO_2 and 1mM methyl viologen. The brei was transferred into a test-tube and incubated at 25°C. The reaction was started by the addition of 0.2ml of 10mg/ml sodium dithionite in 95mM sodium bicarbonate. Controls consisted of sodium bicarbonate minus the sodium dithionite. The assay was stopped after 30 minutes by vigorous aeration of the assay mix which oxidised the blue reduced methyl viologen to the colourless leuco form. 30µl of the assay solution was then added to 970µl H_2O followed by 1ml of 1% sulphanilamide in 3N HCl and 1ml 0.02% NED. Full colour development occurred within 15 minutes after which the optical density was read at 540nm (Snell and Snell, 1949). The amount of NO_2 reduced was calculated from a previously established standard curve (0-100 nmoles KNO_2, (Fig 2.3)).

2.4.5. Protein determination

Protein was determined according to Bradford, (1976). The reagent consisted of 100mg Coomassie blue G-250, 55ml 96% ethanol, 110ml orthophosphoric acid made up to a volume of 1 litre with H_2O, and filtered twice through Whatman No.1 filter paper.

5ml of reagent was added to 0.1ml of appropriately diluted sample and mixed. After full colour development, which occurred within 10 minutes, the optical density was read at 595nm. Calibration curves (0-100µg protein) were prepared with BSA as standards, (Fig 2.4).

2.4.6. Nitrate determination

Nitrate was determined using the method described by Bright *et al.*, (1983). The reagent solution was prepared by adding an equal volume of concentrated Analar sulphuric acid to concentrated Analar orthophosphoric acid, in a highly exothermic reaction. Once cool the reagent solution is stored in a dark bottle for at least one week before use.

When ready for use 0.5% (w/v) diphenylamine sulphonic chromogene, (Szechrome NAS®) was added to the reagent solution in a tightly sealed bottle and stirred until the powder dissolved and the liberated gas absorbed (up to 3 hours), producing a colourless solution. Nitrate content was determined by the addition of 5ml of the reagent to 0.5ml of prediluted sample containing about 0.1µmoles nitrate, followed by thorough mixing by

Fig 2.4. Protein standard curve.

The plot shows a typical calibration curve demonstrating the direct relationship between standard amounts of bovine serum albumin and optical density at 595nm after the addition of Bradfords reagent, over the range 0-100μg protein.

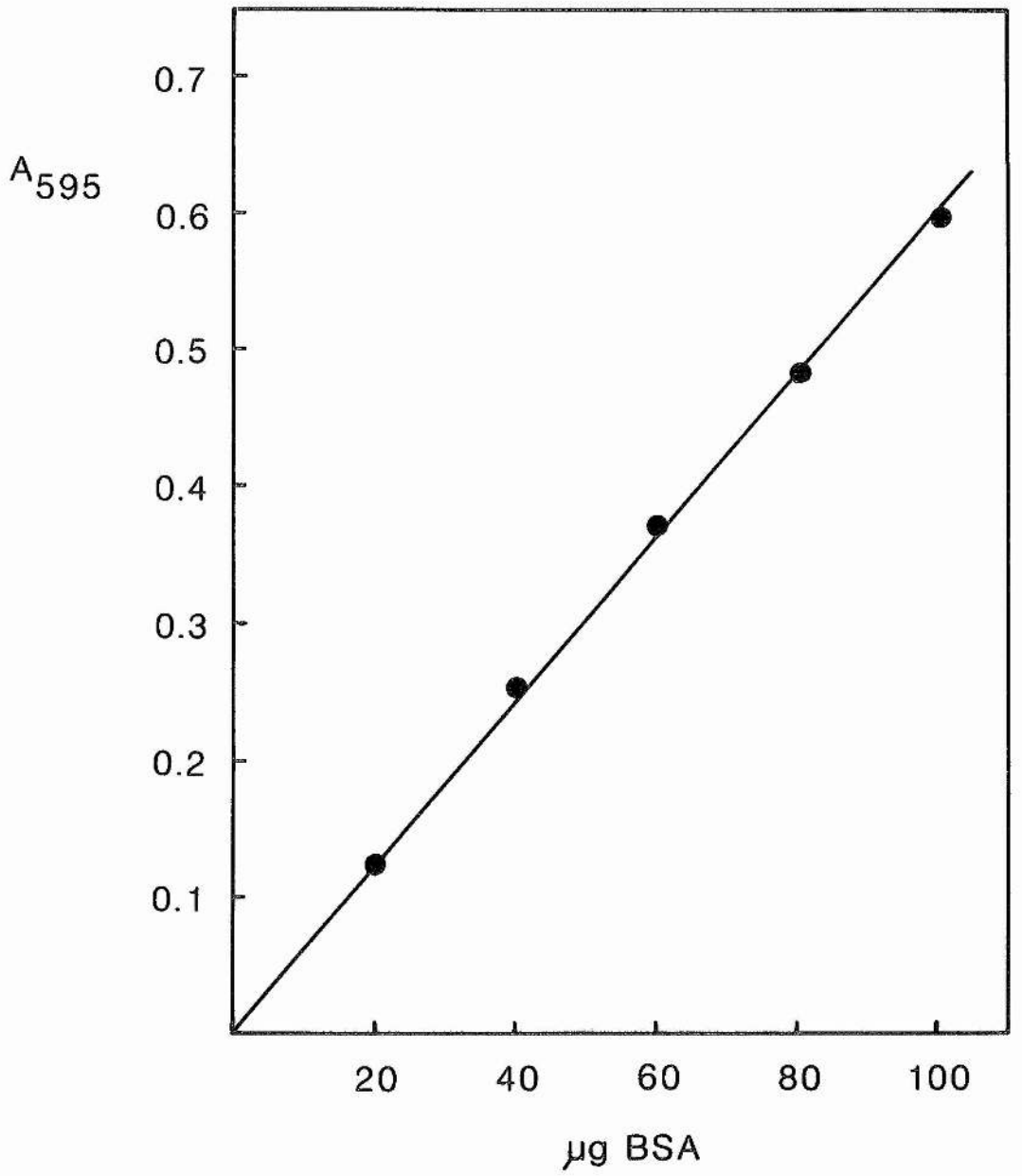
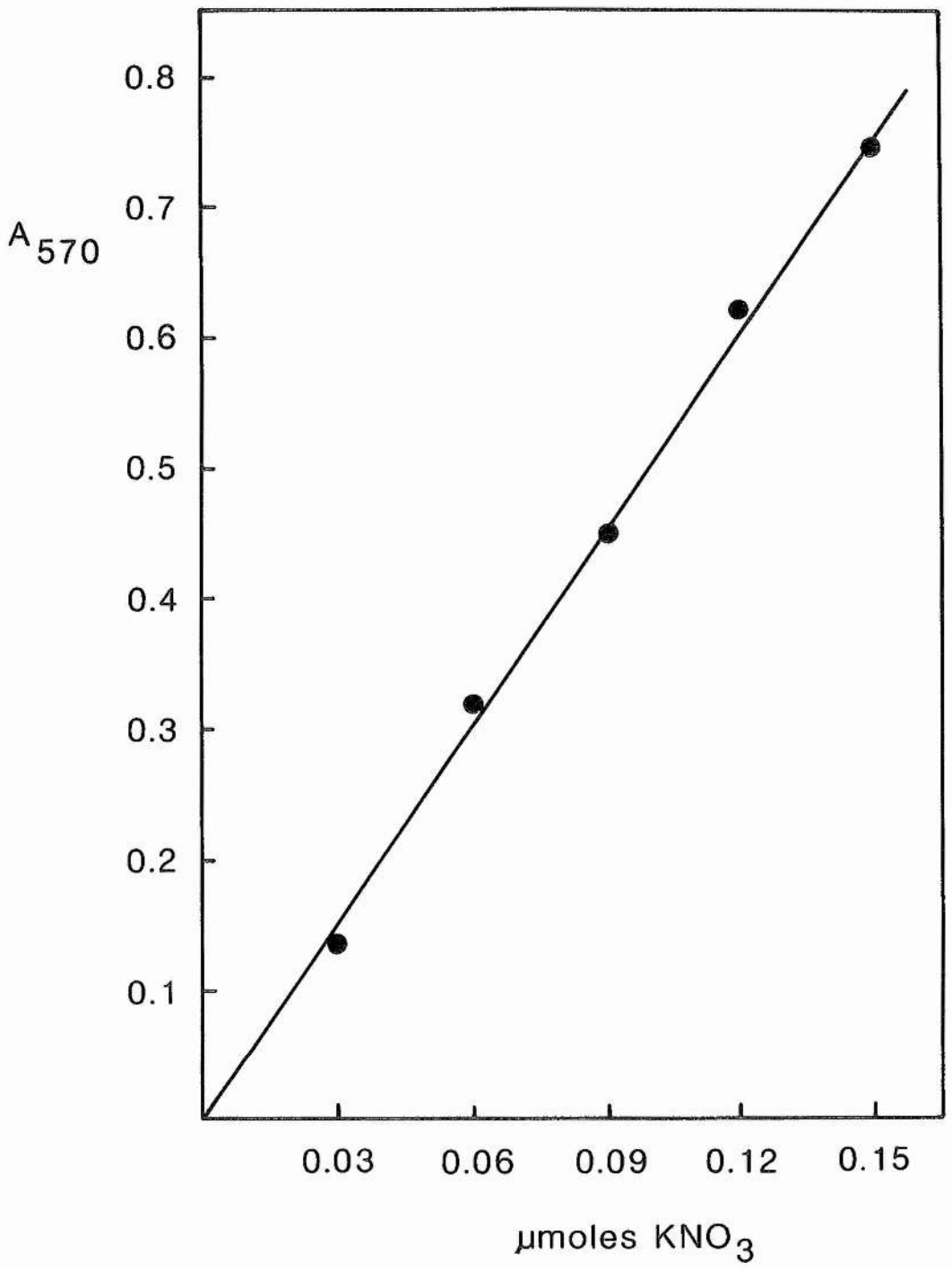

A_{595}
0.7
0.6
0.5
0.4
0.3
0.2
0.1
20
40
60
80
100
µg BSA

Fig 2.5. Nitrate standard curve.

The plot shows a typical calibration curve demonstrating the direct relationship between standard amounts of nitrate and optical density at 570nm after the addition of Szechrome NAS®, over the range 0-0.15µmoles nitrate.


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
A570
0.03
0.06
0.09
0.12
0.15
µmoles KNO3

inversion. The optical density was read at 570nm within one hour and the nitrate determined from a calibration curve (0-0.15μmoles KNO_3 (Fig 2.5)).

2.4.7. Non-denaturing polyacrylamide gel electrophoresis

Vertical slab gels contained 10% (w/v) acrylamide and 0.27%(w/v) methylene-bisacrylamide in 0.1M Tris-glycine buffer pH 8.9, 240μl of 250mg/ml ammonium persulphate and 0.025% (v/v) TEMED. The reservoir buffer was 0.1M Tris/glycine pH 8.9. The gel was pre-equilibrated prior to sample loading for 30 minutes at a constant voltage with an initial current of 40mA. Samples (up to 200μg protein, (60-100μl tissue extract)) were mixed with 20μl loading buffer (2mg% bromophenol blue, 10% glycerol) prior to loading on to the gel. Electrophoresis was performed at 4^0C for 4 hours at a constant voltage with an initial current of 40mA.

2.4.8. Gel staining for nitrite reductase activity

Gels to be stained for nitrite reductase activity were first equilibrated in 50mM potassium phosphate buffer pH 7.5 for 30 minutes at 4^0C, followed by incubation in 90ml staining solution (50mM potassium phosphate pH 7.5, 2mM KNO_3, 1mM methyl viologen). The methyl viologen was reduced by addition of 10ml of 10mg/ml sodium dithionite in 0.29M sodium bicarbonate, and then placed under a nitrogen atmosphere in a dessicator for up to 2 hours until clear bands of activity appeared in the blue gel. On exposure to the air the methyl viologen is oxidised and the blue colour fades from the gel within 15 minutes. A permanent record can be made by either photography or by developing the gel in 2.5% (w/v) triphenyl tetrazolium chloride, which produces an insoluble nonautoxidisable formazan (Hucklesby and Hageman, 1973). Nitrite reductase activity is then seen as a colourless band in the gel which is otherwise stained a permanent red colour.

2.4.9. SDS polyacrylamide gel electrophoresis

Vertical slab gels were prepared essentially as described by Laemmli, (1970). The separating gel contained 10% acrylamide, 0.25% methylene-bisacrylamide, 0.375M Tris pH 8.8, 12% sucrose, 0.1% SDS, and was polymerised by the addition, to a final concentration, of 0.1% ammonium persulphate and 0.0005% TEMED. The stacking gel contained 4.8% acrylamide, 0.13% methylene-bisacrylamide, 0.175M Tris pH 6.8, 12% sucrose, 0.08% SDS, and was polymerised by the addition, to a final concentration, of 0.08% ammonium persulphate and 0.0005% TEMED.

Samples were prepared for loading by adding an equal volume of 2X sample buffer (0.12M Tris pH 6.8, 4% SDS, 20% glycerol, 10% mercaptoethanol and 2mg% Bromophenol blue) to the sample in test tubes and placing them in a boiling water bath for 2 minutes. The tubes were removed and allowed to cool for 5 minutes before loading on to the gel.

The reservoir buffer contained 0.025M Tris pH 8.3, 0.192M glycine, and 0.1% SDS. Electrophoresis was carried out at 10mA for 5 hours and then 2mA overnight.

2.4.10. Protein electrotransfer (western blotting)

Proteins were transferred from the acrylamide gels onto nitrocellulose filters, essentially as described by Towbin *et al.*, (1979). The gel was held against the nitrocellulose in a sandwich of 3MM filter papers and Scotchbrite pads held in place by a plastic surround placed in the electrotransfer tank.

The tank contained transfer buffer (20mM Tris pH 8.3, 193mM glycine, 20% methanol) and transfer was performed at a constant current of 250mA for 3 hours.

2.4.11. Development of electroblots (western blots) for nitrite reductase cross reacting material

All incubations and washes of the nitrocellulose filters were performed in shallow trays placed on a gently rocking table. The nitrocellulose filters were incubated in trays containing excess blocking buffer (10mM Tris buffer pH 8.0, 150mM NaCl, 0.05% Tween-20 (TBST) containing 4% Marvel) for 60 minutes. This solution was replaced with 50ml TBST containing 1% Marvel and polyclonal nitrite reductase antiserum (up to 1:50,000 dilution), and left overnight. The filters were then washed with three changes (20 minutes each) of excess (80-100ml) TBST and then incubated with alkaline phosphatase-linked anti-rabbit IgG (1:1000 dilution) in 50ml TBST containing 1% Marvel for 2 hours. The filters were again washed with three changes (20 minutes each) of excess TBST (80-100ml). The nitrocellulose filters were then developed with nitroblue tetrazolium (33mg%) and 5-bromo-4-chloro-3-indolyl phosphate (disodium salt, 10.5mg%) in 80ml 100mM Tris pH 9.5, 100mM NaCl, 5mM $MgCl_2$. The alkaline phosphatase enzyme converts the BCIP to a blue indigo which releases hydrogen ions that reduces the NBT salt to the corresponding, intensely purple, diformazan. Both these compounds are deposited at the site of alkaline phosphatase activity, producing purple bands at the protein-antibody recognition site. The reaction was stopped by thoroughly washing the filter with excess distilled water.

2.5. Immunolocalisation of nitrite reductase

2.5.1. Fixation of tissue

Leaf tissue, sliced into $1mm^2$ pieces with a razor blade, was incubated in 2.5% glutaraldehyde in 0.2M potassium phosphate buffer pH 7.3 for 1-2 hours. The glutaraldehyde solution was removed with a Pasteur pipette and the tissue washed in 0.2M potassium phosphate buffer pH 7.3 (2x15 minutes). Tissue was then either post-fixation stained with 1% osmium tetroxide for 1 hour, washed in 0.2M potassium phosphate buffer pH 7.3 (2x15 minutes) or taken straight to the embedding stage.

2.5.2. Embedding of tissue

The tissue was dehydrated through an alcohol series, with two 10 minute incubations in 25, 50, and 75% ethanol followed by four 10 minute incubations in 100% ethanol. The tissue was then left overnight in a 1:1 mixture of 100% ethanol:resin (LR White medium grade) with constant movement on a rotating wheel. The following day the ethanol/resin mixture was changed for neat resin and after 8 hours incubation, the resin was changed again and left overnight on the rotating wheel.

The tissue was then transferred, with a Pasteur pipette, to lids taken from polymerisation capsules. The lids were then filled with resin and covered with a glass cover slip, taking care to exclude any air bubbles, as oxygen interferes with the polymerisation process. The blocks were then polymerised by placing them in an oven at $60^{\circ}C$ for 24 hours.

2.5.3. Grid coating

Nickel grids were coated with Formvar by firstly dipping clean glass microscope slides in a solution of 3% (w/v) Formvar in chloroform, which were then allowed to dry, covering the slide with a film of Formvar. Once dry the Formvar film was scored with a scalpel blade then after "huffing" on the scored edge the slide was quickly, but gently, lowered into a container of water allowing the water to seep under the film at the scored edge, thus floating the film off the slide onto the surface of the water.

The grids were then gently placed on top of the film using a pair of fine forceps. The film was then lifted from the surface of the water by placing a piece of paper, cut to be slightly larger than the Formvar film carrying the grids, over the film. Once the paper was

completely wet it was quickly lifted off the water with a pair of forceps, carrying the film and grids with it. Once the paper and film were dry the grids were ready for use.

2.5.4. Microtome sectioning

Ultrathin sections were cut from the polymerised blocks using a Riechart OU40 microtome, with hand made glass knives. 80-120nm thick sections (silver/gold in colour) were lifted on to Formvar coated nickel grids by capillary attraction.

2.5.5. Immunolabelling of ultrathin sections

All incubations and washings of sections were performed by floating the grids (section side down) on drops (5-10µl) of solution. To prevent evaporation all incubations were carried out on pieces of parafilm placed in a petri dish which also contained moist filter papers. Free protein-binding sites on the sections were blocked with 2mg/ml BSA for 1 hour, then incubated with polyclonal nitrite reductase antiserum (1:100 dilution) for 1.5 hours. The grids were then washed with 0.2M potassium phosphate buffer pH 7.5, 1M NaCl, 0.01% Tween-20 ((PBST), 10x1 minute). After incubation with goat anti-rabbit IgG conjugated to 15nm gold aggregates (GARG-15), at a 1 in 20 dilution for 2 hours, the grids were washed in PBST (10x1 minute) and H_2O (10x1 minute).

2.5.6. Post-embedding staining and electron microscopy

The grids were stained with saturated uranyl acetate for 15 minutes, washed with H_2O (10x1 minute) followed by staining with lead citrate (Reynold, 1963) for 15 minutes and again washed with H_2O (10x1 minute). The grids were allowed to dry and then viewed under the electron microscope.

Chapter 3

Selection of nitrite accumulating barley plants

3.1. Introduction

3.1.1. Mutant analysis

Mutant analysis has classically been used to study genetic and biochemical regulation of metabolic pathways in micro-organisms, with large bacterial and fungal populations screened relatively easily for individuals lacking specific enzyme activities or products. Isolation and characterisation of mutant alleles defining genetic loci is also a powerful tool in the study of metabolism for plant geneticists and biochemists.

The selection of biochemically defined mutants in higher plants is complicated by the genetic and biochemical complexity, and the long generation time of these organisms. Crucial to overcoming these problems is the ability to induce high mutation frequencies within a population and the availability of suitable screening procedures allowing the identification of the desired mutant phenotype amongst the wild type individuals.

3.1.2. Chemical mutagenesis

High frequencies of mutations can be produced in populations of leaf protoplasts or cell suspensions using chemical mutagens (ethylmethane sulphonate, N-methyl-N'-nitro-nitrosoguanidine and N-ethyl-N-nitrosourea) or ionising radiations (X-rays and gamma-rays). Regeneration of these cell lines to whole plants is not yet possible for all plant species, particularly monocotyledons, making analysis under normal biochemical and physiological conditions impossible. The possibility also exists that certain mutations may not be expressed in the artificial environment of cell tissue culture, and therefore cannot be selected, mutations likely to be involved in regulatory aspects of cell metabolism being particularly important.

The isolation and characterisation of whole plant mutants is therefore of great importance. Homozygous recessive mutations may be induced within second (M_2) generations of self pollinating plants by treatment of the first (M_1) generation with chemical mutagens (ethylmethane sulphonate and sodium azide) and ionising radiation (Blonstein and King, 1986).

Sodium azide has been used in the study reported here to induce homozygous mutations in populations of barley. Sodium azide yields high mutant frequencies in barley compared to γ–radiation (7.25 per cent chlorophyll-deficient seedlings compared to 0.97 per

cent on a M_2 seedling basis, equivalent to 63 per cent and 11.7 per cent on a M_1 spike basis respectively) (Kleinhofs *et al.*, 1978).

Inorganic azide mutagenicity is mediated through a metabolically synthesised organic azide, L-azidoalanine, formed by the action of O-acetylserine (thiol)-Lyase, (EC 4.2.99.8) using O-acetylserine and azide as substrates (Ciesla *et al.*, 1980), with azide substituting for the natural substrate, sulphide, in the reaction. Azide mutagenesis is highly attenuated by a deficient excision-repair system (Kleinhofs and Smith, 1976), suggesting the formation of a premutation lesion recognisable by the excision-repair mechanism. Mutagenesis appears to proceed from this by a "direct mispairing" mechanism (Owais and Kleinhofs, 1988).

The requirement of metabolic activation by O-acetylserine (thiol)-Lyase explains the lack of azide mutagenicity in animal systems that do not possess this activity. Thus, azide has been used more often in plant systems (Owais and Kleinhofs, 1988). Azide has been found to increase mutant frequencies in barley (Nilan *et al.*, 1973), peas (Sander and Muelbauer, 1977), rice (Awan *et al.*, 1980), maize (Conger and Carabia, 1977), soybean (Vig, 1973) and petunia (Khalatkar and Kashikar, 1980), but was found to be nonmutagenic in some species, notably *Arabidopsis* (Gichner and Veleminsky, 1977).

Selection procedures need to be simple, quick and inexpensive to allow large numbers of individuals to be tested. Common selection procedures make use of the knowledge of the biochemistry of the pathway. The use of metabolic analogues as positive selection agents, with mutations conferring resistance to the toxic effects of the analogue, or a metabolic derivative, makes identification of the mutant phenotype a simple task. This approach has been very successful in the isolation of nitrate reductase-deficient mutants in higher plants with the use of chlorate as a positive selection agent (Oostindier-Braaksma and Feenstra, 1972; Braaksma and Feenstra, 1982a,b). The rationale behind the use of chlorate is that nitrate reductase reduces chlorate to the toxic compound chlorite which accumulates and kills the plant. Individuals which, for whatever reason, lack a catalytically functional nitrate reductase would be expected to be chlorate resistant and could be selected as such.

Where positive selection agents are not available, mutants can be selected by adoption of a non-selective total isolation procedure (Warner *et al.*, 1977) where each plant within a population is tested for expression of the mutant phenotype. This approach is more labour intensive than positive selection schemes since it requires the processing of every

plant within the mutagenised population. However, prudent choice of the selection procedure may still allow significant numbers of individuals to be processed.

3.1.3. Nitrite reductase-deficient mutants

The first plant variants defective in nitrite reduction were described in *Haploppapus gracilis* by Gilissen *et al.*, (1985). They selected a callus (Ao) which was able to grow in the presence of normally toxic levels of asparagine. After X-irradiation, this line was plated on medium containing alanine as nitrogen source and calli growing at wild type rates were selected. Two of these lines failed to grow on medium containing nitrate as a nitrogen source and excreted nitrite. While *in vitro* nitrate reductase activity was similar to the wild type, both *in vivo* nitrate reductase and *in vitro* nitrite reductase activities were only 20-40 per cent of the wild type level. Attempts at plant regeneration were unsuccessful and the genetic basis of the defects is unknown.

Clearly whole plant mutants defective in nitrite reduction are of particular significance since they allow both the characterisation of the number and type of genes which determine this step in nitrate assimilation, and also the consequences of such mutations in the intact differentiated organism. A further consideration is that some types of mutation may only be expressed, and thus can only be selected, at the whole plant level.

A non-selective total isolation procedure has been used in this study to isolate individuals within M_2 populations of barley, treated with azide in the M_1 generation, that are deficient in nitrite reduction. The rationale behind the selection scheme is that mutants should be identifiable by virtue of the fact that they would be expected to accumulate nitrite after exposure to nitrate (Dunn-Coleman *et al.*, 1984; Wray, 1986).

Analysis of such mutants should allow both the identification of genetic loci whose existence can be deduced from a knowledge of the biochemical characteristics of the pathway and also of genes encoding previously unknown components of the pathway. A possible constraint anticipated is the reportedly toxic effect of nitrite to higher plants (Joy, 1969b; Chroboczek Kelker and Filner, 1971; Wray, 1986). This may be overcome by a transient exposure of the populations to nitrate thus limiting the amount of nitrite accumulated by the mutant plants.

3.2. Results

3.2.1. Selection of nitrite accumulators

M_2 populations of barley seed, treated with sodium azide in the M_1 generation and either collected in bulk or carried on M_1 spikes were screened for nitrite accumulation after exposure to nitrate. Under the screening conditions used wild type plants did not accumulate nitrite and as anticipated most batches were nitrite negative (Fig 3.1). Where batches produced a positive nitrite reaction, the plant or plants responsible were identified upon rescreening of individual plants in the batch (Fig 3.1). There were no instances of false positives amongst the batches screened as nitrite accumulating individuals were identified from every nitrite accumulating batch.

Over 250,000 M_2 seedlings derived from bulk-harvested M_1 plants and 94,000 M_2 seedlings from 30,000 M_1 spikes were screened for nitrite accumulation, identifying 30 M_2 nitrite accumulating seedlings (including 4 chlorophyll-deficient seedlings) at an overall frequency of about 1:12,000 (Table 3.1).

The frequency of nitrite reduction-deficient mutants varies considerably between the M_2 bulk-harvested seed and M_2 seed collected as M_1 spikes, with averages of 1:17,000 and 1:6,300 respectively, (Table 3.1). The increase in frequency between the bulk and spike-harvested M_2 populations for nitrite accumulators reflects increases in the sodium azide concentration used in the mutagenic treatment of the M_1 generations. Bulk-harvested seed was treated in the M_1 generation with 1mM sodium azide compared to the spike-harvested seed (except cv. Corniche and Digger) which were treated with 2mM sodium azide (Table 3.2), demonstrating the concentration effect of azide in the M_1 generation upon the M_2 generation mutation frequency.

Fig 3.1. Identification of nitrite accumulating seedlings.

7 day old (4 days germination in the dark followed by 2 days growth in the light plus 16-20 hours treatment with 25mM KNO_3) M_2 seedlings were screened for nitrite accumulation in batches of about 50 seedlings by removing 5-10mg tissue from each seedling in the batch and grinding the pooled tissue in a mortar and pestle with 1ml water, 1ml 1% sulphanilamide in 3N HCl and 1ml 0.02% NED. The presence of nitrite produces a characteristic pink azo dye thus enabling the identification of batches containing a nitrite accumulator. The spikes within the nitrite accumulating batch were then screened separately to identify the spike carrying the nitrite accumulator(s). Each seedling from the identified spike was then rescreened to select the seedling(s) responsible for the nitrite accumulation. Seedlings derived from bulk-harvested batches were rescreened individually directly after identification of a nitrite accumulating batch.

1. Test 341, batch 3 (nitrite non-accumulator); 2. Test 341, batch 4 (nitrite accumulator); 3. Batch 4, spike IX 1009 (nitrite non-accumulator); 4. Batch 4, spike IX 1010 (nitrite accumulator); 5. Spike IX 1010, selection I (nitrite accumulator); 6. Spike IX 1010, selection II (nitrite accumulator); 7. Spike IX 1010, selection III (nitrite non-accumulator); 8. Spike IX 1010, selection IV (nitrite non-accumulator); 9. Spike IX 1010, selection V (nitrite non-accumulator).

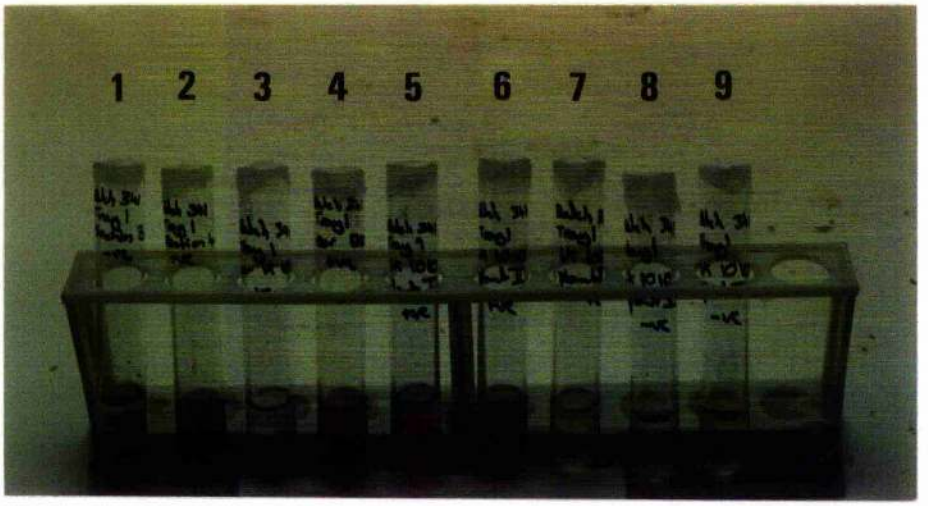

1 2 3 4 5 6 7 8 9

Table 3.1. Number and type of M_2 barley seed (bulk or spike-harvested) screened for nitrite accumulation.

Cultivar	No. screened		Number of	Frequency	
	spikes	seed	accumulators	spikes	seed
Golden Promise	≈	118,547	5	≈	1:23,709
Apex	≈	8,421	-	≈	-
Maris Mink	≈	1,234	-	≈	-
Klaxon	≈	41,767	3	≈	1:13,922
Patty	≈	83,693	7	≈	1:11,956
Golden Promise	6,188	23,390	3	1:2,063	1:7,797
Tweed	5,000	19,357	1	1:5,000	1:19,357
Natasha	3,968	11,022	2	1:1,984	1:5,511
Vista	4,706	13,550	-	-	-
Klaxon	5,000	13,699	4	1:1,250	1:3,425
Doublet	1,176	1,060	1	1:1,176	1:1,060
Digger	1,448	5,862	2*	1:724	1:2,931
Corniche	2,991	6,040	2*	1:2,991	1:3,020

≈ : bulk harvested

* : chlorophyll-deficient

7 day old (4 days germination in the dark followed by 2 days growth in the light plus 16-20 hours treatment with 25mM KNO_3) M_2 seedlings (bulk or spike-harvested), were screened for leaf tissue nitrite accumulation.

Table 3.2. Effect of M_1 azide concentration on M_2 population germination rate and frequency of nitrite accumulators and chlorophyll-deficient seedlings.

Cultivar	M_1[NaN_3] (mM)	$\%M_2$ germination	$\%M_2$ nitrite accumulators	$\%M_2$ chlorophyll-deficient
Golden Promise	1	≈	0.004	1.38
Apex	1	≈	-	3.70
Mink	1	≈	-	4.62
Klaxon	1	≈	0.007	3.46
Patty	1	≈	0.008	1.86
Digger	1	60	0.034	4.77
Corniche	1	53	0.033	4.78
Golden Promise	2	81	0.013	1.67
Tweed	2	80	0.005	1.21
Natasha	2	58	0.018	1.02
Vista	2	61	-	0.75
Klaxon	2	60	0.029	2.31
Doublet	2	19	0.094	1.70

≈ : not determined

Populations of 7 day old (4 days germination in the dark followed by 2 days growth in the light plus 16-20 hours treatment with 25mM KNO_3) M_2 barley seedlings (bulk or spike-harvested) derived from M_1 plants treated with various concentrations of sodium azide in the M_1 generation were analysed for germination rate, nitrite accumulating seedlings and for chlorophyll-deficient seedlings.

3.2.2. Selection growth

3.2.2.1. Bulk-harvested selections

Plants expressing the nitrite accumulation phenotype were rescued from the trays of nitrate-treated vermiculite and placed in a hydroponic growth system with glutamine as the sole nitrogen source.

A total of 15 plants were identified as nitrite accumulators from the screening of the bulk-harvested M_2 populations. 5 plants grew to maturity in the hydroponic system (Fig 3.2) and produced M_3 seed (Table 3.3 and Fig 3.3). Of the remaining 10 plants which failed to produce M_3 seed, 2 plants were sterile (Fig 3.3), while 8 plants failed to survive beyond 40 days (Table 3.3 and Fig 3.3).

3.2.2.2. Spike-harvested selections

The initial screening of populations of M_2 seed derived from M_1 spikes produced 15 M_2 seedlings expressing the nitrite accumulation phenotype (including 4 chlorophyll-deficient seedlings) from 12 M_1 spikes. The remaining 175 M_2 seed carried on the 12 M_1 selected spikes were sown. The 136 M_2 seedlings which germinated were screened for nitrite accumulation. This resulted in a further 5 M_2 seedlings (including one chlorophyll-deficient seedling) from 4 M_1 spikes expressing the mutant phenotype. This gave an overall total of 20 nitrite accumulating seedlings (including 5 chlorophyll-deficient seedlings (Table 3.4)).

All selections were rescued from the trays of nitrate-treated vermiculite and maintained in the hydroponic growth system. 6 plants, from 5 spikes, grew to maturity and produced M_3 seed (Table 3.4 and Fig 3.3). The other 9 green selections failed to produce M_3 seed due either to sterility, or premature death (Fig 3.3) during hydroponic growth of the M_2 selections (Table 3.4).

In general, the growth characteristics of M_2 selections in the hydroponic growth system were noticeably different from those of the wild type controls. Selected plants grew less vigorously, producing fewer tillers and were generally smaller than the controls (Fig 3.4). There were appreciable differences in the rate of growth, with M_2 selections taking much longer to reach maturity and flower, increasing the generation time (Fig 3.4).

Selected M_2 nitrite accumulators produced low numbers of M_3 progeny seed when allowed to self pollinate (Table 3.3 and 3.4). While many of the M_2 spikes failed to produce

Fig 3.2. Maintenance of nitrite accumulating plants in hydroponic culture.

Plants identified as nitrite accumulators were rescued from the trays of nitrate-treated vermiculite and maintained in an environmentally controlled hydroponic growth system, with 1mM glutamine as the sole nitrogen source. Surviving plants were grown to maturity and self pollinated seed harvested. 1: wild type control, 2: Golden Promise 10/3.

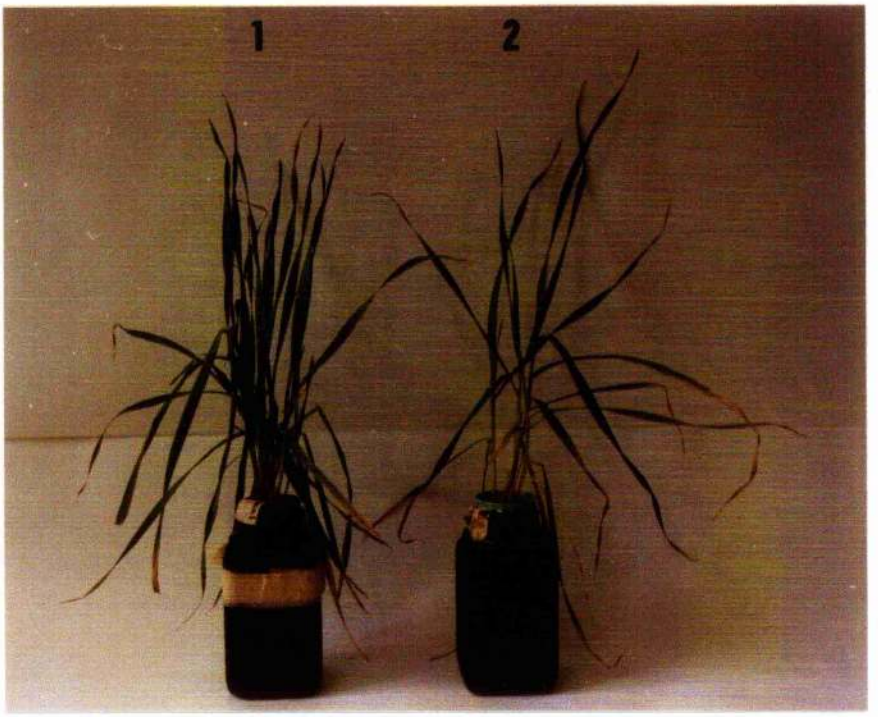

1
2

Table 3.3. Survival, growth and M_3 seed setting of bulk-harvested M_2 selections.

Isolation code	survival time (days)	M_3 seed	Average M_3 seed set (seed/spike)
Golden Promise 1/1	36	0	-
Golden Promise 10/3	337	209	11.0
Klaxon 33/4	384	357	10.2
Klaxon 45/5	14	0	-
Golden Promise 92/6	188	19	3.8
Golden Promise 93/7	294	0	-
Patty 97/8	184	0	-
Patty 106/9	183	461	1.5
Klaxon 121/10	12	0	-
Patty 133/11	35	0	-
Golden Promise 138/12	193	86	5.4
Patty 229/13	40	0	-
Patty 229/14	4	0	-
Patty 229/15	40	0	-
Patty 231/16	16	0	-

Bulk-harvested seedlings identified as nitrite accumulators after treatment with nitrate were transferred to the hydroponic growth system and maintained on 1mM glutamine as the sole nitrogen source. Surviving fertile plants were allowed to self pollinate to produce M_3 seed.

Fig 3.3. Production of M_3 self pollinated seed from M_2 nitrite accumulating selections.

Azide mutagenesis and nitrite accumulation affected recovery of self pollinated M_3 seed from nitrite accumulating selections, causing sterility within mature selections or premature death. A: Golden Promise 138/12. Normal growth in hydroponic culture producing self pollinated M_3 seed. B: Golden Promise 93/7. Weak growth in hydroponic culture producing sterile, mature plant. C: Golden Promise 4169 V, VI, VII. Premature death in young seedlings, seedlings V and VII died shortly after selection.


A


B

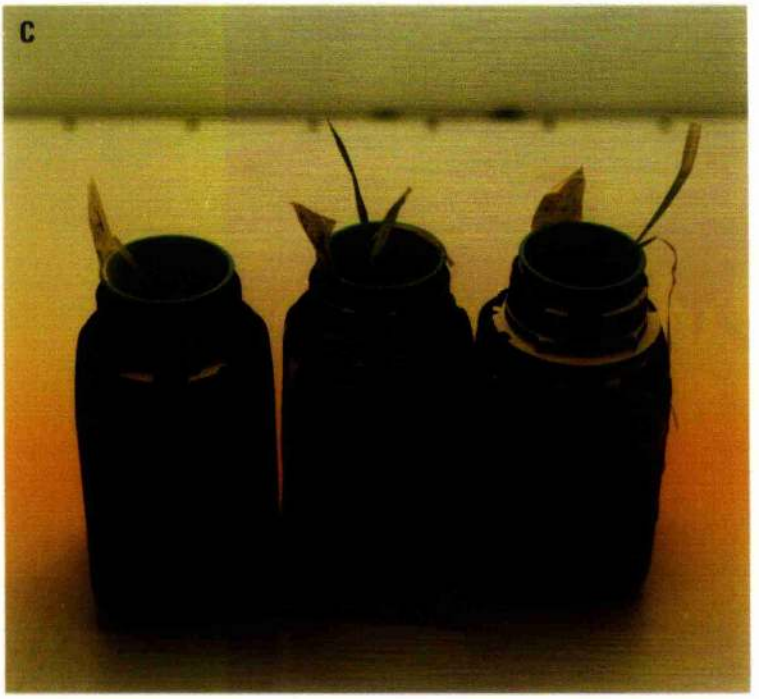

C

Table 3.4. Survival, growth and M_3 seed setting of M_2 spike-harvested selections.

Isolation code	survival time (days)	M_3 seed	Average M_3 seed set (seed/spike)
Corniche 897 I	0*	0	-
Corniche 897 II	0*	0	-
Corniche 897 III	0*	0	-
Doublet 603 I	5	0	-
Digger 679 I	0*	0	-
Digger 870 I	0*	0	-
Klaxon 48 I	679	4	2.0
Golden Promise 2406 I	M	97	6.9
Golden Promise 4169 I	209	0	-
Golden Promise 4169 II	363	0	-
Golden Promise 4169 VI	183	0	-
Golden Promise 4169 VII	31	0	-
Tweed 3999 II	244	12	4.0
Klaxon 1010 I	491	20	3.3
Klaxon 1010 II	58	0	-
Klaxon 2760 I	285	5	2.5
Klaxon 2760 VI	151	8	8.0
Natasha 1489 I	14	0	-
Natasha 1489 VI	1	0	-
Natasha 2114 I	156	0	-

M : selection maintained

* : chlorophyll-deficient

Spike-harvested seedlings identified as nitrite accumulators after treatment with nitrate were transferred to the hydroponic growth system and maintained on 1mM glutamine as the sole nitrogen source. Surviving fertile plants were allowed to self pollinate to produce M_3 seed.

Fig 3.4. Growth characteristics of nitrite accumulating selections in hydroponic culture.

Tweed 3999 II and Tweed wild type control plant were sown simultaneously and maintained in the hydroponic growth system. Tweed 3999 II demonstrates the abnormal growth exhibited by the majority of selected plants in the hydroponic growth system. Growth rate was slower compared to wild type control plants and selected plants were generally smaller and produced fewer tillers.

IT WT: Tweed wild type, IT 3999: Tweed 3999 II, M_2


I T WT
I T 3999

any seed the average seed set by M_2 nitrite accumulators was 6.2 seed/spike (Table 3.3 and 3.4) compared to the average of about 30 seed/spike for wild type controls. A doubling in the M_1 azide concentration caused a reduction in the average M_2 seed set per spike from 8.3 (1mM azide) to 4.9 (2mM azide).

3.2.2.3. M_3 progeny growth

M_3 progeny from all 5 bulk-harvested selections could be grown in compost in the greenhouse where they were watered daily with tap water (Fig 3.5). These plants grew strongly and produced M_4 seed (Fig 3.5).

M_3 progeny from spike-harvested selection Tweed 3999 II were unable to grow in compost (Fig 3.6). The seedlings withered and died within 3 weeks after transfer into compost.

3.2.3. Chlorophyll-deficient mutants

3.2.3.1. Frequency of chlorophyll-deficient seedlings

The frequency of chlorophyll-deficient seedlings in the spike-harvested M_2 populations exhibits an inverse relationship with the concentration of azide used in the mutagenic process of the M_1 populations of seed (Table 3.2). The average frequency for chlorophyll-deficient M_2 seedlings derived from M_1 populations treated with 1mM sodium azide was 3.51 per cent compared to 1.47 per cent for M_2 chlorophyll-deficient seedlings derived from M_1 populations treated with 2mM sodium azide (Table 3.2).

3.2.3.2. Nitrite accumulating chlorophyll-deficient seedlings

1,743 M_2 chlorophyll-deficient seedlings present in the screened M_2 spike-harvested populations were also screened for nitrite accumulation. 5 M_2 selections derived from 3 M_1 spikes were found to accumulate nitrite (Table 3.4). However, as expected, these plants were unable to survive beyond the one-leaf stage of development.

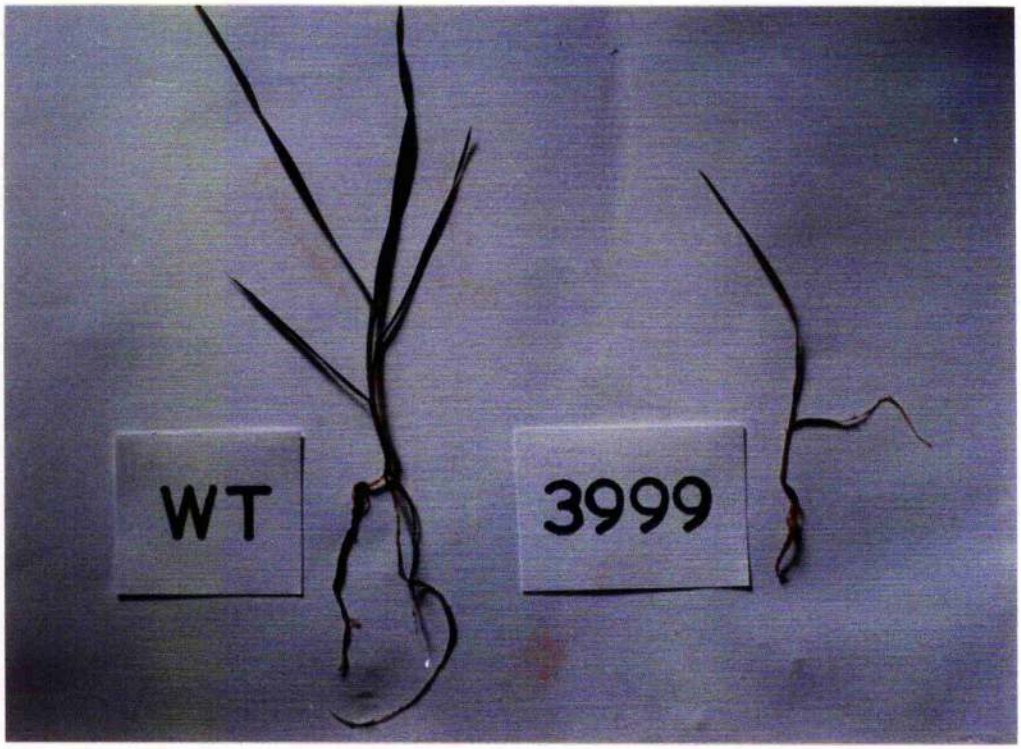
Fig 3.5. Growth of M_3 progeny, derived from bulk-harvested selection Golden Promise 10/3.

M_3 progeny seed from bulk-harvested selections were sown in pots of compost, placed in the greenhouse and watered daily with tap water. The seedlings grew vigorously into healthy mature plants and produced M_4 seed.

Fig 3.6. Growth of M_3 progeny, derived from spike-harvested selection Tweed 3999 II.

A 7 day old M_3 progeny seedling from selection Tweed 3999 II was transferred to compost and grown under constant light and watered with half-strength Hoaglands nutrient solution minus nitrate. The plant grew poorly and within 3 weeks of transfer to compost had withered and died.

WT: Tweed wild type, 3999: Tweed 3999 M_3


WT
3999

3.3. Discussion

3.3.1. Nitrite accumulating selections

The overall frequency with which nitrite accumulators were identified within M_2 populations of barley (1:12,000) was lower than those found for other phenotypes expressed within M_2 populations of barley, particularly nitrate reductase-deficient mutants which were identified at frequencies of 1:1,200 and 1:900 (Kleinhofs *et al.*, 1978) and 1:700 (Warner *et al.*, 1977). Nitrate reductase mutants from M_2 populations of barley screened for chlorate resistance were identified at an overall frequency of 1:7,400 (Bright *et al.*, 1983).

The disparity in frequency of isolation of nitrate reductase-deficient barley seedlings and nitrite accumulating seedlings in azide treated populations of barley may be due to some form of specific action by azide, or its derivatives, in the mutagenic process. Azide exhibits a remarkable specificity in the *Salmonella typhimurium* bacterial system (Owais and Kleinhofs, 1988) with only the *his* G46 mutation showing reversion mutations with high frequency while other *his* mutations, including *ochre*, *amber* and unspecified missense mutations could not be reverted at all (Owais and Kleinhofs, 1988). Azide was highly effective in inducing forward (5-fluorouracil resistant) mutations in a *uvr* A strain, but was unable to induce Trp^+ revertants in the same strain under identical treatment conditions (Mckee *et al.*, 1979). Thus, azide appears to have a different specificity than either UV and alkylating agent type mutagens (Owais and Kleinhofs, 1988). However, the nature of the specific action of azide, or its derivatives, in *Salmonella typhimurium* has not yet been elucidated. It is also unclear whether azide, or its derivatives, interact with specific sequences of DNA during the mutagenic process in higher plants.

The screening procedures would allow for some of the differences in frequencies as nitrate reductase-deficient M_2 plants were selected when expressing up to 10 per cent *in vivo* nitrate reductase activity, so allowing the selection of "leaky" mutants. Nitrite reduction-deficient seedlings containing "leaky" type mutations may be present in the screened populations but these individuals would only be identified if the block was "tight" enough to allow an overall accumulation of nitrite.

3.3.2. Maintenance of selected nitrite accumulators

Selected nitrite accumulators could not be maintained on nitrate since it is likely that nitrogen would not be assimilated into amino-N due to the block in the pathway. Thus, the plants would suffer from nitrogen starvation while nitrite levels may continue to increase to toxic levels, either event proving lethal to the plant. Therefore, the selected lines were maintained in the hydroponic growth system with 1mM glutamine as nitrogen source.

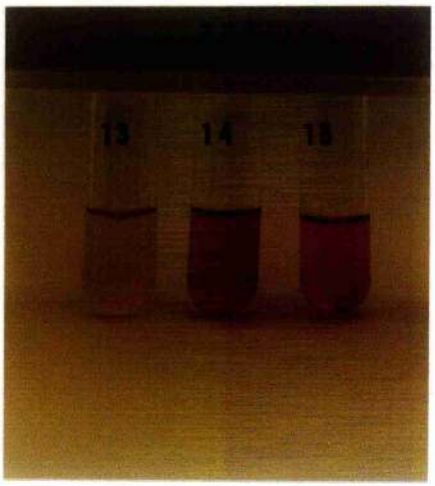
Maintenance of the selected M_2 nitrite accumulators remained a constant problem with selections exhibiting abnormal growth characteristics in the hydroponic growth system (less vigorous and slower growth, and poor seed setting) or failing to survive. The loss of 66 per cent of the original selections from both the bulk and spike-harvested populations may be due to several factors. Seedlings that failed to survive showed little signs of growth beyond the germination stage and within 1-2 weeks began to wither. This suggests an inability to maintain a metabolic rate sufficient for growth and survival beyond the early stages of germination and growth where the seedling continues to draw upon seed reserves. These seedlings exhibited rapid withering and bleaching of their leaves (Fig 3.3) perhaps due to the toxic effects from nitrite accumulation.

The amount of nitrite accumulated by the selections varied (estimated at 0.3-10μmol/gm fresh weight.) with some of the selections accumulating higher levels of nitrite failing to survive (Fig 3.7). The variation in the amounts of nitrite accumulated by the individual selections may be a result of different types of mutation producing either a total block, or a leaky mutation allowing some nitrite reduction and so reducing the amount accumulated. Alternatively, the selections may have different metabolic rates, either naturally or as a result of the mutagenic process, which could result in variations in the amount of nitrite produced and thus accumulated.

Microbial contamination of the nutrient solution was a major problem, particularly during the early stages of plant development when the selections appeared to be most susceptible to contamination of the root systems. Wild type control seedlings grew more vigorously than the selections, with microbial contamination much less of a problem, suggesting microbial contamination of the root systems was a consequence of poor plant growth as well as a contributing factor. This problem diminished once the selection had established itself and produced a more substantial root system. However, maintenance of the selections continued to be a major, labour-intensive task.

Fig 3.7. Leaf tissue nitrite content of selected nitrite accumulators.

Batch 229 from the bulk-harvested screen contained 3 seedlings that accumulated nitrite (left to right Patty 229/13, 229/14, 229/15). The amount of nitrite accumulated differed between the 3 seedlings (Patty 229/14 accumulated the highest level of nitrite) and correlated with the survival of the seedlings (Patty 229/14 survived only 4 days after isolation compared to 40 days for Patty 229/13 and 229/15, Table 3.3).


13
14
15

The deleterious effects on subsequent generations derived from azide treated populations of barley has been a continual problem in the isolation of mutant plants. Bright *et al.*, (1983) report that from 33 plants selected for chlorate resistance from azide-treated barley populations, only 2 selections produced progeny derived from self pollination. However, the progeny from these 2 fertile selections appeared to be wild type and represented false positives from the chlorate resistance screen. Warner *et al.*, (1977) reported that 56 per cent of nitrate reductase-deficient selections from an azide-treated barley population failed to grow and produce seed or were sterile. These results suggest a general negative side-effect on the metabolism and vigour of M_2 populations of barley treated with azide in the M_1 generation which is most likely due to random mutations within the genome. Thus, the weak growth and poor seed setting of selected nitrite accumulators is probably due to the azide treatment and not a direct effect of the screening procedure, or the defect responsible for the nitrite accumulation. However, these general effects are likely to be exacerbated in plants selected for nitrite accumulation by the microbial contamination of the nutrient solutions and also the accumulation of potentially harmful levels of nitrite within the plant tissues.

3.3.3. Chlorophyll-deficient mutants

Increases in the sodium azide concentration used to treat the M_1 populations caused increases in the frequency of green nitrite accumulators identified within the M_2 generations, as stated above. The average frequency of chlorophyll-deficient seedlings in M_2 generations, derived from M_1 populations treated with 1mM sodium azide (3.51 per cent), is comparable to reports elsewhere, 4.06 per cent (Nilan, 1981), 7.12 per cent (Kleinhofs *et al.*, 1978). However, the frequency of chlorophyll-deficient seedlings in the M_2 generations appears to exhibit an inverse relationship to the concentration of azide used during the mutagenesis of the M_1 generation.

Nilan, (1981) reported that increases in the azide concentration (from 10^{-5} to 10^{-3}M) used in the treatment of M_1 populations of barley seed produced increases in the frequency of chlorophyll-deficient seedlings (from 0.28 to 4.06 per cent) within the M_2 generation. In the studies reported here the frequency of chlorophyll-deficient M_2 seedlings in populations screened for nitrite accumulation is reduced as the M_1 azide concentration is increased beyond 1mM. Doubling the M_1 azide concentration from 1mM to 2mM produces a decrease in the average frequency of chlorophyll-deficient seedlings in the M_2 generation from 3.51 to

1.47 per cent, while the overall frequency of nitrite accumulating seedlings increased two fold (from 0.017 to 0.032 per cent, Table 3.2).

The decrease in the number of chlorophyll-deficient mutants may be the result of increases in mutations elsewhere within the genome. Tuleen *et al.*, (1968) described the selection and genetic analysis of a group of 44 revertant barley plants induced by chemical mutagens ethyl methanesulphonate (EMS) and triethylene melamine (TEM) and ionising radiation (X-rays). 32 of these mutations represented a minimum of 17 different loci responsible for the suppression of chlorophyll deficiency producing quantitative alterations in several major chloroplast pigments modifying the phenotypic effects towards the wild type.

5 M_2 selections from 3 M_1 spikes expressed the double phenotype of nitrite accumulation and chlorophyll deficiency. It is possible to envisage a single mutational event producing both phenotypes, since nitrite reductase is dependent upon photosystem I, via ferredoxin, for its reducing power. However, the possibility that the phenotypes are due to unrelated mutations within the same genome still exists.

Chapter 4

Biochemical characterisation of selected nitrite accumulators

4.1. Introduction

4.1.1. Biochemical analysis of selected nitrite accumulators

The biochemical characterisation of the selected lines is an important step in mutant analysis allowing functions to be assigned to the mutant alleles defining genetic loci.

A wide variety of mutations might be expected to block nitrite reduction and so lead to an accumulation of nitrite within the leaf tissue, viz, within the nitrite reductase structural gene affecting either catalytic function or the transit peptide (Back *et al.*, 1988); in processing of the precursor protein; formation and insertion of prosthetic groups (mutation in the *Escherichia coli cys* G locus affects sirohaem synthesis (MacDonald and Cole, 1985)); in recognition of the chloroplast/plastid (a protein receptor mediating import of the *rbc* S gene product has been identified (Pain *et al.*, 1988)); in electron donation and in some aspect of regulation.

Of greater importance, perhaps, would be the identification of functions necessary for nitrite reduction that cannot be deduced from a knowledge of the pathway. Since no whole-plant mutants defective in nitrite reduction have previously been isolated in higher plants, mutations affecting such unexpected loci would be of great interest.

4.2 Results

4.2.1. Analysis of M_2 selections

Analysis of M_2 nitrite accumulators was complicated by their slow and weak growth in the hydroponic growth system (section 3.2.2.). It was not possible to remove gram quantities of tissue to perform quantitative analysis without compromising the growth and survival of the plants. Therefore, limited analysis was performed on some M_2 selections once techniques for the analysis of milligram quantities of leaf tissue had been developed.

4.2.2. M_2 bulk-harvested selections

No biochemical data is available for nitrite accumulating M_2 seedlings selected from screened M_2 populations derived from bulk-harvested seed, since the techniques for the analysis of milligram quantities of leaf tissue (vacuum infiltration with nitrate, total-extract *in vitro* nitrate reductase and nitrite reductase assays and western blotting) to allow removal and analysis of tissue without compromising the survival of the selected plant were not established at this time.

4.2.3. M_2 spike-harvested selections

4.2.3.1. Nitrite reductase activity

Milligram amounts of leaf tissue removed from M_2 selections and vacuum infiltrated with nitrate possessed very low to zero levels of total-extract *in vitro* nitrite reductase activity. The activities measured were within the range 0-18 µmoles nitrite reduced/gm fresh weight/hour (Table 4.1), about 10 per cent of the wild type control. Leaf tissue from one selection (Klaxon 48) possessed about 60 per cent of the wild type control levels of total-extract *in vitro* nitrite reductase activity (52 µmoles nitrite reduced/gm fresh weight/hour, Table 4.1).

4.2.3.2. Nitrate reductase activity

Leaf tissue removed from M_2 selections and vacuum infiltrated with nitrate possessed levels of total-extract *in vitro* nitrate reductase that were comparable with wild type controls with an average value of about 5.0 µmoles nitrite produced/gm fresh weight/hour (Table 4.1).

Table 4.1. M_2 leaf tissue total-extract *in vitro* nitrite reductase and nitrate reductase activities and *in vivo* nitrate reductase activity after vacuum infiltration with nitrate.

selection	*in vitro* activities		*in vivo* activity
	NiR µmol/gm/hr	NR µmol/gm/hr	NR µmol/gm/hr
Golden Promise wt	110	4.5	5.9
Golden promise 2406 V	6	4.5	2.0
Golden Promise 4169 II	18	2.7	2.8
Klaxon wt	86	7.6	4.4
Klaxon 48 I	52	4.8	3.1
Klaxon 1010 I	0	4.0	N.D.
Klaxon 2760 I	6	6.9	5.6

N.D. : not determined

Leaf tissue (about 10mg) of similar age and development was removed from M_2 selections (grown on 1mM glutamine in the hydroponic growth system) and vacuum infiltrated with 0.1M KNO_3. After 16-18 hours incubation in the light the tissue was assayed for total-extract *in vitro* nitrite reductase and nitrate reductase activities and *in vivo* nitrate reductase activity.

Leaf tissue from M_2 selections possessed *in vivo* nitrate reductase activities that were comparable with wild type control values with an average of about 4.5 μmoles nitrite produced/gm fresh weight/hour (Table 4.1).

4.2.3.3. Nitrite reductase cross reacting material

As nitrite accumulating M_2 seedlings were identified, leaf tissue (5-10 mg) was removed for analysis of nitrite reductase cross reacting material by western blotting. The leaf material therefore originated from 7 day old nitrate-treated plants (germinated in the dark for 4 days, grown in the light for 2 days and treated with 25mM KNO_3 for 16-20 hours).

5 M_1 spikes carrying 8 nitrite accumulating and 29 nitrite non-accumulating seedlings were analysed for nitrite reductase cross reacting material. Leaf tissue from all 8 nitrite accumulating seedlings lacked detectable nitrite reductase cross reacting material (Fig 4.1 and Table 4.2). All nitrite non-accumulating M_2 seedlings, with the exception of Golden Promise 4169 V, contained nitrite reductase cross reacting material (Fig 4.1 and Table 4.2).

Nitrite accumulating seedlings were transferred to the hydroponic growth system (section 3.2.2.). The surviving selections were rechecked for leaf tissue nitrite reductase cross reacting material 16-20 hours after vacuum infiltration of leaf tissue with nitrate. All plants that had previously lacked nitrite reductase cross reacting material as 7 days old seedlings continued to lack nitrite reductase cross reacting material after further growth in the hydroponic growth system (Fig 4.2).

3 M_2 Selections (Golden Promise 4169 VI, Golden promise 2406 V and Klaxon 48 I), that had been maintained in the hydroponic growth system, and were not analysed for leaf nitrite reductase cross reacting material as 7 day old seedlings were also analysed after vacuum infiltration of leaf tissue with nitrate. Golden Promise 2406 V lacked detectable nitrite reductase cross reacting material, but Klaxon 48 I contained levels that were comparable with wild type controls (Fig 4.2 and Table 4.2.).

Figure 4.1. Leaf nitrite reductase cross reacting material in M_2 seedlings carried on selected M_1 spikes.

7 day old seedlings from M_1 spikes identified as carrying *in vivo* nitrite accumulators were analysed for leaf tissue nitrite reductase cross reacting material. Leaf tissue (about 10mg) was removed from each seedling carried by the selected M_1 spike and after micro-extraction the extract was subjected to SDS-PAGE and western blotting.

A. Lane 1: GP4169 I*, lane 2: GP4169 II*, lane 3: GP4169 III, lane 4: GP4169 IV, lane 5: GP4169 V**, lane 6: GP4170 I.

B. Lane 1: IT 3998 I, lane 2: IT 3999 I, lane 3: IT 3999 II*, lane 4: IT 3999 III, lane 5: IT 3999 IV, lane 6: IT 3999 V, lane 7: IT 4000 I.

C. Lane 1: IX 1009 I, lane 2: IX 1010 I*, lane 3: IX 1010 II*, lane 4: IX 1010 III, lane 5: IX 1010 IV, lane 6: IX 1010 V, lane 7: IX 1011 I.

D. Lane 1: IX 2759 I, lane 2: IX 2760 I*, lane 3: IX 2760 II, lane 4: IX 2760 III, lane 5: IX 2760 IV, lane 6: IX 2760 V, lane 7: IX 2761 I.

E. Lane 1: IX 2760 VI*, lane 2: IX 2760 VII, lane 3: IX 2760 VIII, lane 4: IX 2760 IX, lane 5: IX 2760 X, lane 6: IX 2760 XI, lane 7: IX 2760 XII, lane 8: IX 2760 XIII, lane 9: IX 2760 XIV, lane 10: IX 2760 XV, lane 11: IX 2760 XVI, lane 12: IX 2760 XVII, lane 13: IX 2760 XVIII.

F. Lane 1: IN 1488 I, lane 2: IN1489 I*, lane 3: IN1489 II, lane 4: IN1489 III, lane 5: IN1490 I.

* : nitrite accumulator/NiR-CRM absent

** : nitrite non-accumulator/NiR-CRM absent

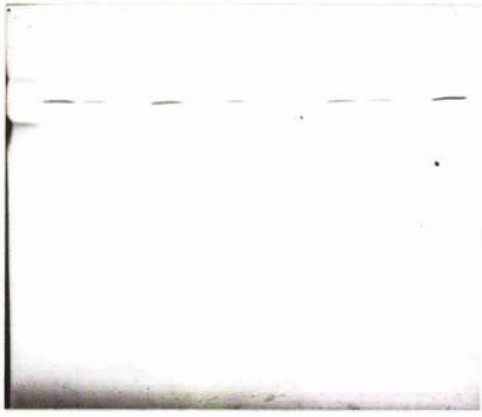
A 1 2 3 4 5 6

B 1 2 3 4 5 6 7

C 1 2 3 4 5 6 7

D 1 2 3 4 5 6 7

E 1 2 3 4 5 6 7 8 9 10 11 12 13



F 1 2 3 4 5

Table 4.2. Leaf tissue nitrite accumulation and nitrite reductase cross reacting material in M_2 spike harvested plants.

selection	nitrite accumulation	NiR-CRM
Klaxon 48 I	+	+*
Golden Promise 2406 V	+	-*
Golden Promise 4169 I	+	-
Golden Promise 4169 II	+	-
Golden Promise 4169 V	-	-
Golden Promise 4169 VI	+	-*
Tweed 3999 II	+	-
Klaxon 1010 I	+	-
Klaxon 2760 I	+	-
Klaxon 2760 VI	+	-
Natasha 1489 I	+	-

+ : Nitrite accumulator/NiR-CRM present

- : Nitrite non-accumulator/NiR-CRM absent

* : Tissue vacuum infiltrated with nitrate after growth in the hydroponic growth system

7 day old M_2 spike-harvested seedlings (4 days germination in the dark followed by 2 days growth in the light plus 16-20 hours treatment with 25mM KNO_3) were screened for leaf *in vivo* nitrite accumulation and analysed for leaf nitrite reductase cross reacting material by western blotting.

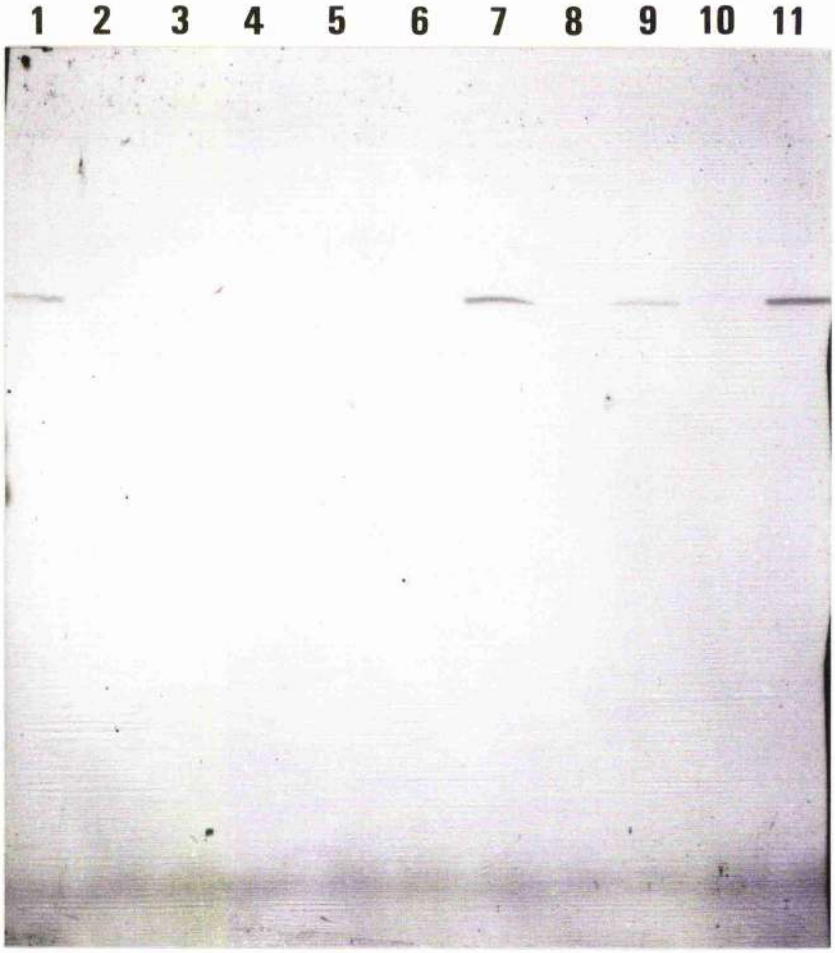
Fig 4.2. Nitrite reductase cross reacting material in leaf tissue from hydroponically grown M_2 selections after vacuum infiltration with nitrate.

M_2 nitrite accumulating plants were maintained in the hydroponic growth system with 1mM glutamine as nitrogen source. Leaf tissue (about 10mg) was removed from healthy plants and vacuum infiltrated with 0.1M KNO_3 and left in the light for 16-20 hours. After micro-extraction the leaf tissue extract was then subjected to SDS polyacrylamide gel electrophoresis and nitrite reductase cross reacting material analysed by western blotting.

Lane 1: Golden Promise wild type, lane 2: Golden Promise 4169 I, lane 3: Golden Promise 4169 II, lane 4: Golden Promise 4169 VI, lane 5: Klaxon 1010 I, lane 6: Tweed 3999 II, lane 7: Klaxon 48 I*, lane 8: Golden Promise 2406 V, lane 9: Digger 1283 I**, lane 10: Klaxon 2760 I, lane 11: Golden Promise wild type.

* : nitrite accumulator/NiR-CRM present

** : nitrite non-accumulator/NiR-CRM present


1 2 3 4 5 6 7 8 9 10 11

4.2.4. Analysis of M_3 progeny derived from bulk-harvested selections

4.2.4.1. *In vivo* nitrite accumulation

5 M_3 progeny seed derived by self pollination of each M_2 selected plant were germinated and grown under the same conditions used in the selection of the nitrite accumulating parents (4 days germination in the dark followed by 2 days growth in the light plus 16 hours treatment with 25mM KNO_3). The 5 seedlings from each line did not accumulate nitrite after analysis for leaf tissue *in vivo* nitrite accumulation (Table 4.3).

4.2.4.2. *In vitro* nitrite accumulation

7 day old M_3 progeny seedlings (5 days germination in the dark followed by 2 days growth in the light watered with half-strength Hoaglands nutrient solution) derived by self pollination of each M_2 selected plant were analysed for leaf tissue *in vitro* nitrite accumulation. 4 M_3 seedlings from each of the 5 selected lines were analysed with every seedling failing to accumulate nitrite after *in vitro* treatment with nitrate (Table 4.3).

4.2.4.3. Nitrite reductase activity

M_3 progeny from all 5 lines expressed a decrease in leaf *in vitro* nitrite reductase activity of about 50 per cent of the wild type control in 7 day old seedlings. Activities varied within the range 3.8-5.5 μmoles nitrite reduced/mg protein/hour compared with the wild type control value of 9.3 μmoles nitrite reduced/mg protein/hour (Table 4.4).

However, when M_3 progeny plants were analysed after 60 days growth in compost under greenhouse conditions of constant light and watered with tap water, leaf *in vitro* nitrite reductase activity levels were comparable with wild type with an average activity of 6.2 μmoles nitrite reduced/mg protein/hour (Table 4.5).

4.2.4.4. Nitrate reductase activity

Leaf *in vitro* nitrate reductase activity levels ranged between 60 and 112 nmoles nitrite produced/mg protein/hour (Table 4.4) and were comparable with the wild type control in 7 day old seedlings.

M_3 progeny grown for 60 days in compost under greenhouse conditions of constant light and watered with tap water expressed wild type levels of leaf *in vitro* nitrate reductase activity (Table 4.5).

Table 4.3. M_3 progeny, derived from bulk-harvested selections, leaf tissue *in vivo* and *in vitro* nitrite accumulation.

selection	nitrite accumulation µmoles nitrite/gm fresh weight	
	in vivo	*in vitro*
Golden Promise wt	0.0	0.0
Golden Promise 10/3	0.0	0.0
Golden Promise 92/6	0.0	0.0
Golden Promise 138/12	0.0	0.0
Klaxon wt	0.0	0.0
Klaxon 33/4	0.0	0.0
Patty wt	0.0	0.0
Patty 106/9	0.0	0.0

M_3 seed, derived from bulk-harvested M_2 selections, were germinated in the dark for 5 days followed by 2 days growth in the light and either watered with 25mM KNO_3 for 16-20 hours and tested for *in vivo* nitrite accumulation or leaf tissue (about 10mg) was removed and floated on 1ml of 0.1M KNO_3 in the light for 16-20 hours and tested for *in vitro* nitrite accumulation.

Table 4.4. M_3 seedling, derived from bulk-harvested selections, leaf tissue *in vitro* nitrite reductase and nitrate reductase activities and nitrate content.

selection	*in vitro* activity NiR µmol/ mgprot/hr	NR nmol/ mgprot/hr	$[NO_3]$ µmol/gm freshwt
Golden Promise wt	9.3	87	7.40
Golden Promise 10/3	5.5	112	11.36
Golden Promise 92/6	3.8	72	12.00
Golden Promise 138/12	4.2	60	10.90
Klaxon 33/4	4.4	74	10.43
Patty 106/9	4.4	81	11.00

M_3 seed, derived from bulk-harvested M_2 selections, were germinated in the dark for 4 days followed by 2 days growth in the light plus 16 hours treatment with 25mM KNO_3. Leaf tissue from the 7 day old seedlings was then assayed for *in vitro* nitrite reductase and nitrate reductase activity and nitrate content.

Table 4.5. Leaf *in vitro* nitrite reductase and nitrate reductase activities in 60 day old M_3 progeny derived from bulk-harvested plants.

selection	*in vitro* activity NiR µmol/mgprot/hr	NR nmol/mgprot/hr
Golden Promise wt	5.8	89
Golden Promise 10/3	6.6	96
Golden Promise 92/6	4.6	85
Golden Promise 138/12	7.1	115
Klaxon wt	5.9	145
Klaxon 33/4	7.5	108
Patty wt	5.7	80
Patty 106/9	5.4	75

M_3 seed derived from bulk-harvested selections were grown in compost in greenhouse conditions of constant light and watered with tap water daily. After 60 days growth leaf tissue from individual plants was assayed for *in vitro* nitrite reductase and nitrate reductase activity.

4.2.4.5. Nitrate accumulation

Leaf tissue nitrate accumulation ranged from 10.43-12.00 μmoles nitrate/gm fresh weight and was on average 50 per cent higher than wild type controls in 7 day old M_3 progeny seedlings (Table 4.4).

4.2.4.6. Nitrite reductase activity staining in non-denaturing polyacrylamide gels

Leaf nitrite reductase activity from the 5 selected lines comigrates with wild type control in non-denaturing polyacrylamide gels stained for nitrite reductase activity (Fig 4.3).

4.2.4.7. Nitrite reductase cross reacting material

Leaf nitrite reductase from the selected lines also comigrates with wild type control leaf nitrite reductase in denaturing SDS polyacrylamide gels, electroblotted onto nitrocellulose filters and detected by monospecific polyclonal antiserum raised against purified barley leaf nitrite reductase (western blotting, Fig 4.4). However, the band intensity for nitrite reductase cross reacting material (detected by western blotting) for leaf extract from 7 day old progeny plants from the selected lines was less than that found for leaf extracts from wild type controls (Fig 4.4).

Figure 4.3. Non-denaturing polyacrylamide gel electrophoresis of leaf nitrite reductase from M_3 progeny derived from bulk-harvested selections.

Leaf tissue from 7 day old (4 days germination in the dark followed by 2 days growth in the light plus 24 hours treatment with 25mM KNO_3) M_3 progeny, derived from bulk-harvested selections, was extracted and subjected to non-denaturing polyacrylamide gel electrophoresis followed by gel staining for nitrite reductase activity.

Lane 1: Golden Promise wild type, lane 2: Golden Promise 10/3, lane 3: Klaxon 33/4, lane 4: Golden Promise 92/6, lane 5: Patty 106/9, Lane 6: Golden Promise 138/12, lane 7: Klaxon wild type. All lanes were loaded with 100µg protein.

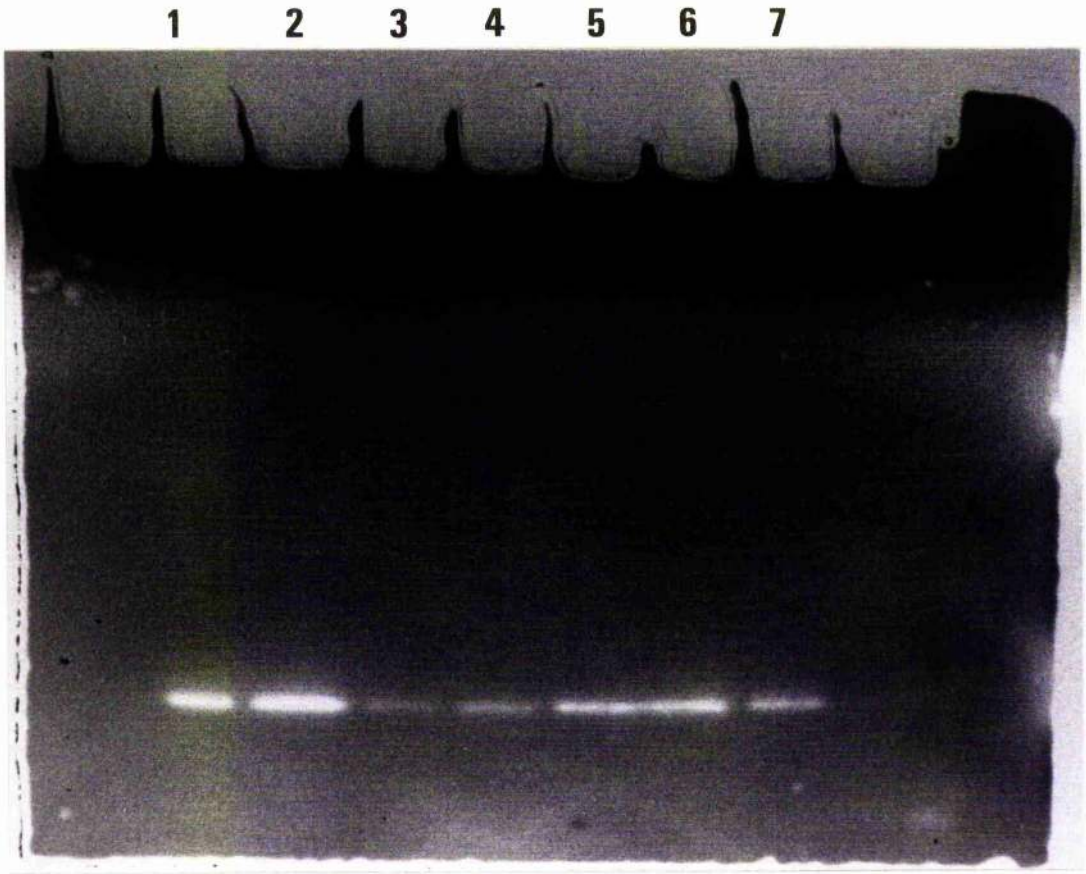

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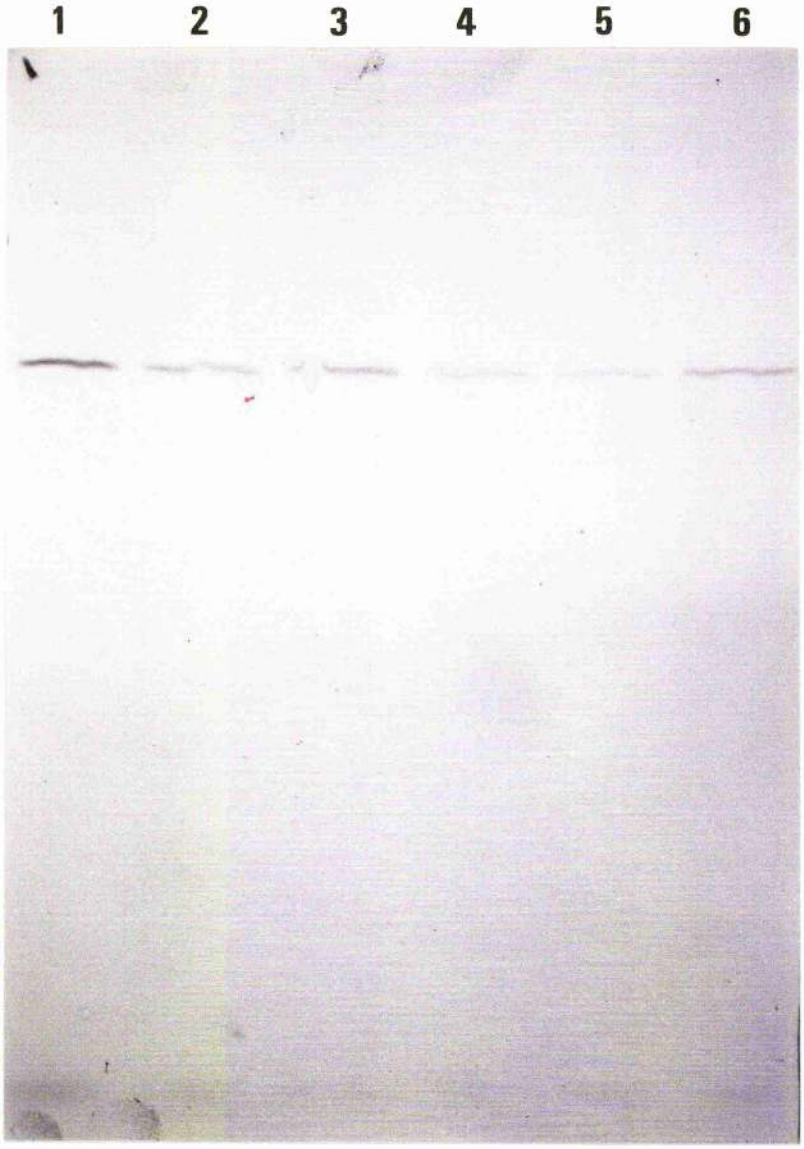
Figure. 4.4. Leaf nitrite reductase cross reacting material in M_3 progeny derived from bulk-harvested selections.

Leaf tissue from 7 day old (4 days germination in the dark followed by 2 days growth in the light plus 16 hours treatment with nitrate) M_3 seedlings, derived from bulk-harvested selections, was extracted and analysed for nitrite reductase cross reacting material by SDS polyacrylamide gel electrophoresis followed by western blotting.

Lane 1: Golden Promise wild type, lane 2: Golden Promise 10/3, lane 3: Klaxon 33/4, lane 4: Golden Promise 92/6, lane 5: Patty 106/9, lane 6: Golden Promise 138/12. All lanes were loaded with 100μg protein.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4.2.5. Analysis of M_3 progeny derived from spike-harvested selections

4.2.5.1. *In vivo* nitrite accumulation

M_3 progeny seed, derived by self pollination of each of the 5 fertile, selected M_2 nitrite accumulating plants, were germinated and grown under the same conditions used for the selection of the nitrite accumulating parents (4 days germination in the dark followed by 2 days growth in the light plus 16 hours treatment with 25mM KNO_3). Seedlings from each line failed to accumulate nitrite in both leaf and root tissue except for progeny derived from Klaxon 1010 I which accumulated nitrite (1.7µmoles nitrite/gm fresh weight) in the leaf tissue (Table 4.6).

4.2.5.2. *In vitro* nitrite accumulation

Leaf tissue from plants maintained in the hydroponic growth system (M_2 selection Golden Promise 2406 V and M_3 progeny derived from Tweed 3999 II and Klaxon 1010 I) was removed and either vacuum infiltrated with 0.1M KNO_3 or simply incubated with 0.1M KNO_3 by floating the tissue on the nitrate solution. After 16-20 hours incubation the tissue was analysed for *in vitro* nitrite accumulation. Leaf tissue from the three selected plants tested exhibited high levels of nitrite accumulation after vacuum infiltration or incubation with nitrate with an average of 5.3µmoles nitrite/gm fresh weight (Table 4.7). Leaf tissue from wild type control plants maintained in the hydroponic growth system did not accumulate nitrite (Table 4.7).

4.2.5.3. Nitrite reductase activity

4.2.5.4. Accuracy of *in vitro* nitrite reductase assay

Low levels of nitrite reductase activity are difficult to measure accurately with the dithionite reduced methyl viologen *in vitro* nitrite reductase assay. Activity is determined by the loss of nitrite from a high initial concentration so that low activities produce small differences in nitrite concentration between the test and control assays. A variety of controls were performed for assays with wild type leaf tissue extract, by omitting either the dithionite or enzyme extract or using enzyme extract that had first been placed in a boiling water bath for 2 minutes, to determine the most accurate assay. The precision of the manipulations necessary to perform the *in vitro* methyl viologen nitrite reductase assay result in a minimum theoretical percentage error of 5.8 per cent. The measured error is comparable with

Table 4.6. Leaf and root tissue nitrite accumulation and nitrite reductase cross reacting material in M_3 progeny derived from spike-harvested selections.

selection	nitrite accumulation		NiR-CRM	
	leaf	root	leaf	root
	µmoles nitrite/gm fresh wt			
Klaxon 48	0.0	0.0	+	+
Golden Promise 2406	0.0	0.0	-	-
Tweed 3999	0.0	0.0	-	-
Klaxon 1010	1.7	0.0	-	-
Klaxon 2760	0.0	0.0	-	-

+ : NiR-CRM present

- : NiR-CRM absent

M_3 seeds derived from self pollination of nitrite accumulating selections were grown under screening conditions (4 days germination in the dark followed by 2 days growth in the light plus 24 hours treatment with 25mM KNO_3). Leaf and root tissue from the 7 day old plants was then screened for *in vivo* nitrite accumulation and analysed for nitrite reductase cross reacting material.

Table 4.7. *In vitro* nitrite accumulation by selected plants grown in the hydroponic growth system after vacuum infiltration or incubation with nitrate.

selection	*in vitro* nitrite accumulation	
	vacuum infiltration	incubation
	µmoles nitrite/gm fresh weight	
Golden Promise wt	0.0	0.0
Golden Promise 2406 V M_2	7.8	6.3
Tweed 3999 M_3	5.8	4.8
Klaxon 1010 M_3	1.8	N.D.

N.D. : not determined

Pieces of leaf tissue from selected plants grown in the hydroponic growth system with 1mM glutamine as nitrogen source were either vacuum infiltrated with nitrate in 1ml 0.1M KNO_3 or incubated with 1ml 0.1M KNO_3, without vacuum infiltration, for 16-20 hours in the light ($115\mu Em^{-2}s^{-1}$). The incubation solution was then tested for nitrite by the addition of 1ml 1% sulphanilamide in 3N HCl and 1ml NED.

the theoretical error in assays performed with leaf tissue extracts from minus-nitrate grown plants with an average error of 5.1 per cent (Table 4.8) but larger in assays performed with leaf tissue extracts from nitrate-grown plants with an average error of 7.5 per cent (Table 4.8).

The average percentage error for assays performed with leaf tissue from minus-nitrate grown plants was larger than the average percentage difference in optical density at 540nm (which determines the loss of nitrite) between the control and test assays (5.1 and 3.3 per cent respectively (Table 4.8)). Thus, the measured percentage error is 155 per cent of the optical density difference as a percentage of the control value (Table 4.8). Therefore, the assay cannot give an accurate measure of such low levels of activity.

The *in vitro* nitrite reductase assay is more accurate when measuring high levels of activity. In assays performed with leaf tissue extracts from nitrate-grown wild type plants the average percentage error was lower than the average percentage difference in optical density at 540nm between the control and test assays (7.5 per cent and 35.5 per cent respectively (Table 4.8)). Thus, the measured percentage error is 21 per cent of the optical density difference as a percentage of the control value (Table 4.8).

The accuracy of measuring low levels of nitrite reductase activity cannot be improved by altering the control assay. In nitrate-grown plants the most accurate (ie lowest total percentage error, 6.3 per cent (Table 4.8)) assay consisted of a control where dithionite was added to reduce the methyl viologen but the enzyme extract was withheld until after the oxidation of the reduced methyl viologen by vigorous aeration. This control was used when assaying leaf tissue extracts from nitrate-grown M_3 progeny for *in vitro* nitrite reductase activity.

4.2.5.5. M_3 progeny nitrite reductase activity

Due to the limited amount of seed derived from the self pollination of the fertile selected M_2 plants, tissue was extracted and assayed from 10 day old seedlings (7 days germination in the dark, 2 days growth in the light plus 24 hours treatment with 25mM KNO_3) to ensure sufficient amounts of plant material were available for analysis.

M_3 progeny from 4 of the 5 selected lines (Golden Promise 2406, Tweed 3999, Klaxon 1010 and Klaxon 2760) possessed very low levels of leaf tissue *in vitro* nitrite reductase activity levels, ranging from 0.6-1.8 μmoles nitrite reduced/mg protein/hour (Table 4.9) which

Table 4.8. Accuracy of the *in vitro* methyl viologen nitrite reductase assay.

assay condition	$O.D._{540}$ ± SD	$O.D._{540}$ (control-test) as percentage of control	Total assay error as percentage of control
A: Minus-nitrate grown plants			
Test +enzyme + dithionite	0.901 ± 0.022	-	-
Control			
1. -enzyme -dithionite	0.935 ± 0.027	3.6	5.3
2. -enzyme +dithionite	0.908 ± 0.032	0.8	5.9
3. +enzyme -dithionite	0.942 ± 0.032	4.4	5.8
4. +boiled enzyme -dithionite	0.946 ± 0.019	4.8	4.4
5. +boiled enzyme +dithionite	0.928 ± 0.017	2.9	4.2

In vitro methyl viologen nitrite reductase assays were performed on leaf tissue extracts from 7 day old barley wild type plants (4 days germination in the dark followed by 2 days growth in the light and treated either with half-strength Hoaglands nutrient solution without nitrate or containing 25mM KNO_3 for 24 hours). Various controls were performed with the enzyme

assay condition	$O.D._{540} \pm SD$	$O.D._{540}$ (control-test) as percentage of control	Total assay error as percentage of control
B: nitrate grown plants			
Test +enzyme + dithionite	0.582 ± 0.025	-	-
Control			
1. -enzyme -dithionite	0.893 ± 0.029	34.8	7.5
2. -enzyme +dithionite	0.895 ± 0.018	35.0	6.3
3. +enzyme -dithionite	0.918 ± 0.037	36.6	8.3
4. +boiled enzyme -dithionite	0.926 ± 0.028	34.4	7.3
5. +boiled enzyme +dithionite	0.921 ± 0.035	36.8	8.1

extract withheld or replaced with enzyme extract that had been placed in a boiling water bath for 2 minutes. Where dithionite was withheld, 95mM sodium bicarbonate was added to the assay. Loss of nitrite was determined by the diazo-coupling reaction with 1% sulphanilamide in 3N HCl and 0.02% NED. Each assay was performed in triplicate and the experiment repeated 4 times, mean results are calculated from all data collected.

were, on average, about 10 per cent of the wild type controls. These measured levels of activity are below the limit of accurate measurement by the dithionite reduced methyl viologen *in vitro* nitrite reductase assay.

M_3 progeny from selection Klaxon 48 expressed a high level of *in vitro* nitrite reductase activity, 12.2 µmoles nitrite reduced/mg protein/hour compared to the wild type control value of 7.8 µmoles nitrite reduced/mg protein/hour (Table 4.9).

4.2.5.6. Nitrate reductase activity

4.2.5.7. NADH-nitrate reductase

Leaf tissue *in vitro* NADH-nitrate reductase activity levels in M_3 progeny from selected lines are very similar to wild type control values (Table 4.9). Nitrate reductase activity levels appear to be dependent upon the cultivar assayed, to some extent at least, with Tweed wild type plants and progeny from Tweed 3999 possessing an activity of about 60 nmoles nitrite produced/mg protein/hour which is about 40 per cent of the activity expressed by wild type plants and selections derived from the cultivars Golden Promise and Klaxon (Table 4.9).

4.2.5.8. Methyl viologen nitrate reductase

M_3 progeny from 4 of the 5 selected lines (Golden Promise 2406, Tweed 3999, Klaxon 1010 and Klaxon 2760) expressed leaf tissue *in vitro* methyl viologen-linked nitrate reductase activity levels very similar to the NADH-nitrate reductase activity levels measured in these lines and in wild type controls (Table 4.9). However, *in vitro* methyl viologen nitrate reductase activity was undetectable in wild type controls and in progeny from selection Klaxon 48 (Table 4.9).

4.2.5.9. *In vivo* nitrate reductase activity

M_3 progeny from nitrite accumulating selections Golden Promise 2406 V and Klaxon 48 I possess *in vivo* nitrate reductase activity (4.5 and 3.2 µmoles nitrite produced/gm fresh weight/hour, respectively) which is comparable with wild type controls (5.9 and 4.4 µmoles nitrite produced/gm fresh weight/hour, respectively).

Table 4.9. M_3 progeny, derived from spike-harvested selections, leaf *in vitro* methyl viologen nitrite reductase, NADH-nitrate reductase, methyl viologen nitrate reductase activities and nitrate content.

selection	*in vitro* activity			
	MV-NiR µmol/ mgprot/hr	NADH-NR nmol/ mgprot/hr	MV-NR nmol/ mgprot/hr	$[NO_3]$ µmol/ gmfshwt
Golden Promise wt	9.4	162	0	7.2
Golden Promise 2406	0.6	165	150	6.9
Tweed wt	9.2	62	0	9.3
Tweed 3999	1.0	61	73	17.6
Klaxon wt	7.8	123	0	10.4
Klaxon 48	12.2	148	0	10.3
Klaxon 1010	1.3	214	224	12.9
Klaxon 2760	1.8	154	135	10.2

Individual M_3 progeny seed derived from M_2 spike screening selections were germinated in the dark for 7 days, grown in the light for 2 days then treated with 25mM KNO_3. After 24 hours the leaf tissue was extracted and assayed for *in vitro* methyl viologen nitrite reductase, NADH-nitrate reductase, methyl viologen nitrate reductase activities and leaf tissue nitrate content.

4.2.5.10. Nitrate accumulation

Leaf nitrate accumulation is very similar to wild type controls for all selected lines except Tweed 3999 which accumulated almost twice the amount of nitrate as the Tweed control (17.6 and 9.3 μmoles nitrate/gm fresh weight respectively (Table 4.9)).

4.2.5.11. ^{15}N-nitrate incorporation into leaf protein

Minus-nitrate grown 7 day old seedlings from selected lines Golden Promise 2406 and Tweed 3999 were treated with 25mM $K^{15}NO_3$ in the light for 24 hours. Leaf protein was precipitated from tissue extracts with trichloroacetic acid and after washing with distilled water and freeze drying was analysed for incorporation of $^{15}NO_3$ into protein at Rothamsted Experimental Station, courtesy of David Powlson and Paul Poulton.

Golden Promise 2406 possessed about 10 per cent and Tweed 3999 possessed about 23 per cent of the wild type control $^{15}NO_3$ incorporation (0.4869 and 1.1077 atom per cent excess compared to 4.8779 and 4.8071 atom per cent excess, respectively (Table 4.10)). The selected lines were analysed first thus eliminating the possibility of carry-over producing artefactual results during the analyses.

4.2.5.12. Nitrite reductase activity in non-denaturing polyacrylamide gels

A functional methyl viologen nitrite reductase activity from leaf tissue extract of M_3 progeny from Klaxon 48 comigrated with the wild type controls in non-denaturing polyacrylamide gels stained for nitrite reductase activity (Fig 4.5). Nitrite reductase activity from leaf tissue extract of M_3 progeny from the remaining 4 lines (Golden Promise 2406, Tweed 3999, Klaxon 1010 and Klaxon 2760) was not detectable in non-denaturing polyacrylamide gels (Fig 4.5).

4.2.5.13. Nitrite reductase cross reacting material

Nitrite reductase cross reacting material was undetectable in leaf and root tissue from M_3 progeny from 4 of the 5 selected lines (Golden Promise 2406, Tweed 3999, Klaxon 1010 and Klaxon 2760 (Fig 4.6 and Table 4.6)) after SDS polyacrylamide gel electrophoresis and western blot analysis. M_3 progeny from selection Klaxon 48 contained nitrite reductase cross reacting material in both leaf and root tissue which comigrated with wild type nitrite reductase in denaturing SDS polyacrylamide gels detected by western blotting (Fig 4.6 and Table 4.6).

Table 4.10. ^{15}N incorporation into leaf protein in selected lines Golden Promise 2406 and Tweed 3999.

selection	nitrate treatment	% N Atom	% excess
GP 2406	25mM $^{15}NO_3$	6.5	0.4869
GP wt	25mM $^{15}NO_3$	6.0	4.8779
GP wt	25mM $^{14}NO_3$	5.6	0.0092
GP wt	25mM $^{14}NO_3$*	6.4	0.0067
IT 3999	25mM $^{15}NO_3$	5.4	1.1077
IT wt	25mM $^{15}NO_3$	5.9	4.8071
IT 3999	25mM $^{14}NO_3$	5.9	0.0317
IT wt	25mM $^{14}NO_3$	7.1	0.0136
IT wt	25mM $^{14}NO_3$*	4.0	-0.0196

* : 25mM $K^{15}NO_3$ added to tissue extract before protein precipitation

7 day old seedlings (4 days germination in the dark followed by 2 days growth in the light watered with half-strength Hoaglands nutrient solution without nitrate) were placed in separate vials containing 5ml of either 25mM $K^{15}NO_3$ or 25mM $K^{14}NO_3$ and maintained in the light for 24 hours. The leaf tissue was then extracted (1:10 wt:vol) in nitrite reductase extraction buffer by grinding in a mortar and pestle. The brei was centrifuged at 30,000g for 15 minutes and the supernatants collected. After protein determinations aliquots of the supernatants containing 0.5mg protein were transferred into separate Eppendorf tubes. An equal volume of cold 10 per cent trichloroacetic acid was added and the tubes left at $4^{\circ}C$ for 30 minutes. The Eppendorf tubes were then centrifuged in a microfuge at 13,000 rpm for 10 minutes after which the supernatant was drained off and the pellet resuspended in 0.5ml distilled water. The protein was washed a further 4 times by trichloroacetic acid precipitation after which the pelleted protein was freeze dried and then sent for ^{15}N analysis.

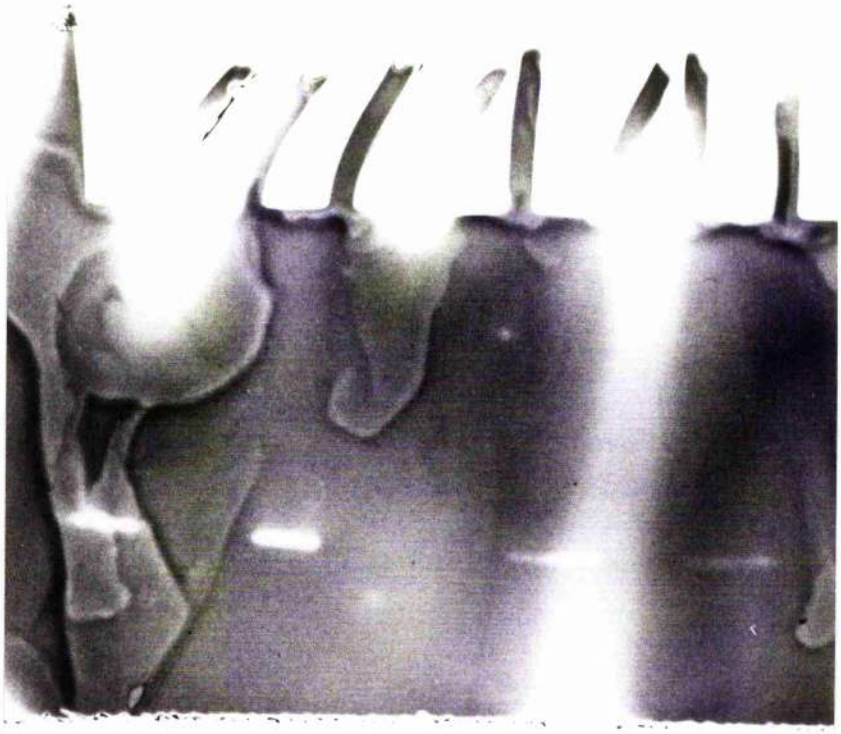
Figure 4.5. Non-denaturing polyacrylamide gel electrophoresis of leaf nitrite reductase from M_3 progeny derived from spike-harvested selections.

Leaf tissue from 7 day old (4 days germination in the dark followed by 2 days growth in the light plus 24 hours treatment with 25mM KNO_3) M_3 progeny, derived from spike-harvested selections, was extracted and subjected to non-denaturing polyacrylamide gel electrophoresis followed by gel staining for nitrite reductase activity.

A. Lane 1: Golden Promise wild type, lane 2: Golden Promise 2406, lane 3: Golden Promise wild type, lane 4: Blank, lane 5: Blank, lane 6: Tweed wild type, lane 7: Tweed 3999, lane 8: Klaxon wild type.

B. Lane 1: Klaxon wild type, lane 2: Klaxon 48, lane 3: Klaxon 1010, lane 4: Klaxon 2760, lane 5: Klaxon wild type, lane 6: Klaxon wild type. All lanes were loaded with 100µg protein.

A 1 2 3 4 5 6 7 8



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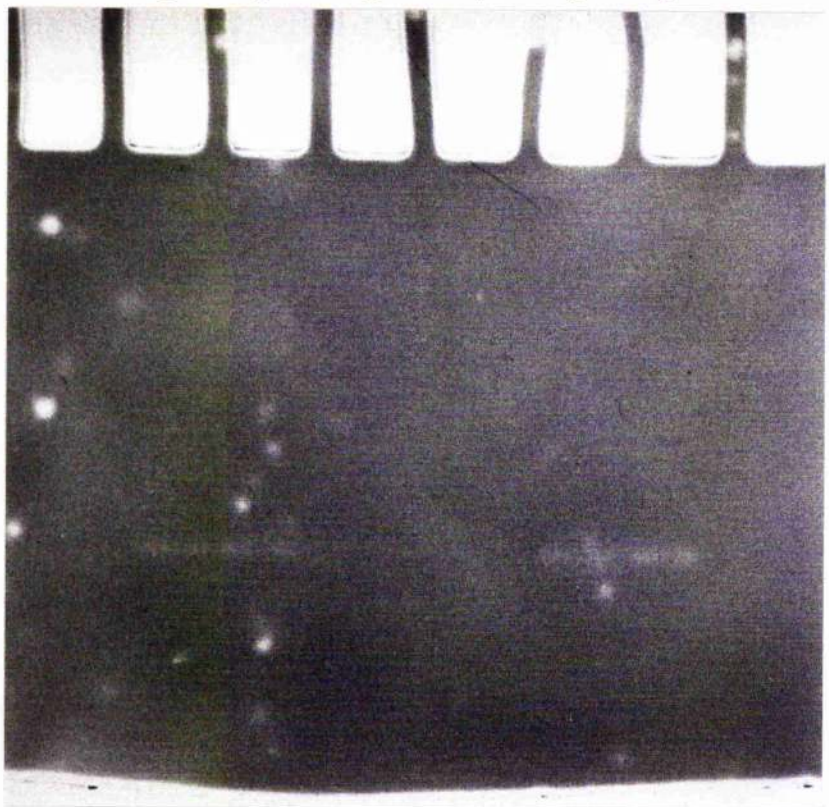

Figure 4.6. Nitrite reductase cross reacting material in leaf and root tissue of M_3 progeny derived from spike-harvested selections.

Leaf and root tissue from 7 day old (4 days germination in the dark followed by 2 days growth in the light plus either 24 hours treatment with 25mM KNO_3) M_3 seedlings, derived from spike-harvested selections, was extracted and subjected to SDS polyacrylamide gel electrophoresis and nitrite reductase cross reacting material analysed by western blotting.

A. Lane 1: Golden Promise wild type (leaf), lane 2: Golden Promise 2406 (leaf), lane 3: Golden Promise wild type (leaf*), lane 4: Golden Promise 2406 (leaf*), lane 5: Golden Promise wild type (root*), lane 6: Golden Promise 2406 (root*), lane 7: Golden Promise wild type (root), lane 8: Golden Promise 2406 (root).

B. Lane 1: Tweed wild type (leaf), lane 2: Tweed 3999 (leaf), lane 3: Tweed wild type (leaf), lane 4: Tweed wild type (root), lane 5: Tweed 3999 (root), lane 6: Tweed wild type (root).

C. Lane 1: Klaxon wild type (leaf), lane 2: Klaxon 48 (leaf), lane 3: Klaxon 1010 (leaf), lane 4: Klaxon 2760 (leaf), lane 5: Klaxon wild type (root), lane 6: Klaxon 48 (root), lane 7: Klaxon 1010 (root), lane 8: Klaxon 2760 (root). All lanes were loaded with 100µg protein.

* : tissue vacuum infiltrated with 0.1M KNO_3

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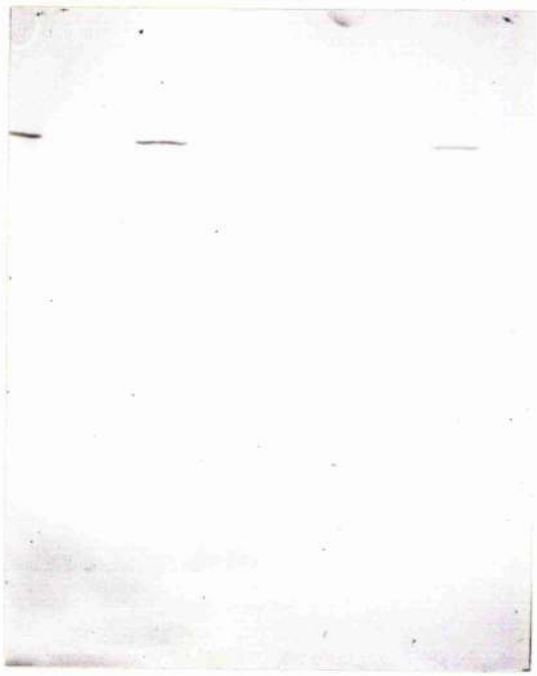
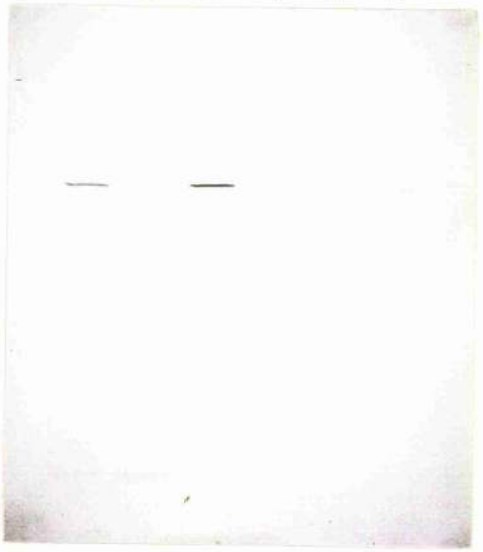
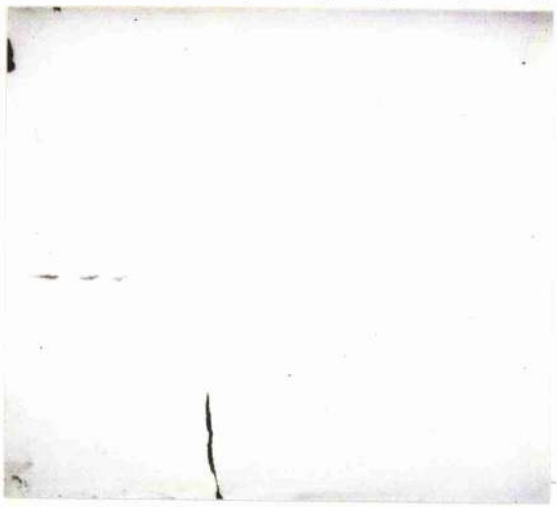


B 1 2 3 4 5 6



C 1 2 3 4 5 6 7 8



4.3. Discussion

4.3.1. Bulk-harvested selections

As stated previously no biochemical data is available on the M_2 selections from the bulk-harvested populations screened for nitrite accumulation. However, M_3 progeny from the 5 surviving, fertile lines did not accumulate nitrite either after *in vivo* or *in vitro* treatment with nitrate. This suggests that these lines do not contain any heritable defect that would cause an accumulation of nitrite after treatment with nitrate.

The M_3 progeny from these lines possess significant amounts of *in vitro* nitrite reductase activity which are about 50 per cent of the level measured in wild type controls in 7 day old seedlings (Table 4.4) and would be more than sufficient to reduce any nitrite formed. The lower levels of nitrite reductase activity are due to lower levels of nitrite reductase protein. Leaf tissue extracts from selected lines possessed lower levels of cross reacting material than wild type controls after western blotting (Fig 4.4). However, after 60 days growth in compost the selected lines possess *in vitro* nitrite reductase activity that is comparable with wild type controls (Table 4.5).

Nitrite reductase, from leaf tissue from the selected lines, was recognised by polyclonal antiserum raised against purified authentic leaf nitrite reductase and was the same molecular weight (Fig 4.4) and expressed the same mobility in non-denaturing polyacrylamide gels (Fig 4.3) as wild type control nitrite reductase activity. Thus, the progeny from selected nitrite accumulators from the bulk-harvested populations screened for nitrite accumulation appear to possess nitrite reductase that is physically similar to wild type controls. However, after treatment with nitrate these plants possess lower levels of nitrite reductase activity compared to wild type due to reduced, but measurable, levels of nitrite reductase protein.

It is unclear whether these observed differences in the regulation of nitrite reductase are due to a mutation specifically affecting the production of nitrite reductase molecules or are due to a general down-regulation of the selected lines due to undefined pleiotropic effects of the azide treatment in the M_1. However, nitrate reductase activities in the selected lines remained unaffected (Table 4.5) suggesting some form of specific effect. Nitrate accumulation was also increased suggesting either an increased rate of accumulation or a decrease in the rate of nitrate assimilation *in vivo*.

4.3.2. Spike-harvested selections

Selected plants from the spike-harvested populations accumulated nitrite after treatment with nitrate due to their inability to produce nitrite reductase protein. M_2 nitrite accumulators tested lacked nitrite reductase cross reacting material (Fig 4.1) and expressed very low to zero levels of total-extract *in vitro* nitrite reductase activity (Table 4.1). One M_2 nitrite accumulating selection (Klaxon 48) possessed wild type levels of nitrite reductase protein molecules (Fig 4.2) and possessed significant levels of total-extract *in vitro* nitrite reductase activity (Table 4.1). Klaxon 48 may represent a false positive isolated from the M_2 screen.

Most M_3 progeny from the 5 fertile lines (Golden Promise 2406, Klaxon 48, Tweed 3999, Klaxon 1010 and Klaxon 2760) failed to accumulate nitrite in leaf or root tissue after *in vivo* treatment with nitrate with only leaf tissue from Klaxon 1010 accumulating nitrite. This suggested that the defect responsible for nitrite accumulation was not inherited by the remaining selected lines. However, in those lines tested, M_3 progeny accumulated nitrite after *in vitro* treatment with nitrate (Table 4.7) and were also defective in the formation of leaf nitrite reductase molecules (Fig 4.6) suggesting a genetic basis for the defect. M_3 progeny from these lines (Golden Promise 2406, Tweed 3999, Klaxon 1010 and Klaxon 2760) possessed low levels of *in vitro* methyl viologen nitrite reductase activity (Table 4.9) although there is evidence to suggest that the dithionite reduced methyl viologen-linked assay may produce artefactual results when measuring low activity levels (section 4.2.5.4.). Thus, the activity measured may not give a true representation of nitrite reductase activity in these plants.

The most compelling evidence suggesting that the *in vitro* methyl viologen nitrite reductase activity observed in these lines is artefactual comes from measurement of dithionite-reduced methyl viologen nitrate reductase activity. This constitutes a coupled assay system since the crude extract from wild type plants should contain both nitrate reductase and nitrite reductase activities, with exogenously supplied nitrate converted to ammonium ions by the sequential action of methyl viologen nitrate reductase activity and methyl viologen nitrite reductase activity. Indeed wild type controls showed an apparent lack of methyl viologen nitrate reductase activity (Table 4.9) presumably due to the action of nitrate and nitrite reductase in converting nitrate to ammonium ions. However, leaf tissue extract from 4 of the selected lines (Golden Promise 2406, Tweed 3999, Klaxon 1010 and

Klaxon 2760) expressed *in vitro* methyl viologen nitrate reductase activities comparable to NADH-nitrate reductase activities (Table 4.9). This demonstrates that methyl viologen nitrite reductase activity was absent from the tissue extract. Therefore, any activity measured in the *in vitro* methyl viologen nitrite reductase assay of the leaf tissue extract from these lines is artefactual. The close similarity between the NADH and methyl viologen nitrate reductase activities (Table 4.9) demonstrates that the rate of reaction of the methyl viologen nitrate reductase activity is similar to NADH-nitrate reductase activity under the assay conditions employed.

Thus, it appears that four lines (Golden Promise 2406, Tweed 3999, Klaxon 1010 and Klaxon 2760) carry a mutation which prevents the formation of nitrite reductase protein, resulting in a loss of nitrite reductase activity. The mutation may occur either in the nitrite reductase structural gene, producing a truncated protein lacking catalytic activity and antigenic sites recognisable by the polyclonal nitrite reductase antiserum, or in a regulatory locus, preventing normal expression of the protein in response to nitrate. Alternatively, the mutation may affect the stability of nitrite reductase molecules. Immediate degradation of newly synthesised nitrite reductase molecules would result in an apparent loss of nitrite reductase protein. The root tissue from these lines also lack detectable nitrite reductase protein (after western blotting) suggesting that formation and/or control of nitrite reductase protein involves the same loci in both tissues.

4.3.3. Sensitivity of *in vitro* nitrite reductase assay

Certain factors suggest that the M_3 *in vitro* methyl viologen nitrite reductase activities may be overstated. The average *in vitro* activity measured for the M_3 plants (excluding Klaxon 48) of 1.2μmoles nitrite reduced/mg protein/hour is similar to the *in vitro* activity for wild type plants grown in the absence of nitrate (section 6.2.2.). Nitrite reductase cross reacting material levels within minus-nitrate grown wild type plants are easily detected by western blotting (section 6.2.2.) while nitrite reductase cross reacting material is undetectable in the M_3 progeny plants even after 24 hours treatment with nitrate.

The assay, while simple in principle, is complex in practice, and requires several manual operations which limit the accuracy of measurement to 5.8 per cent. Since the assay depends upon measurement of the loss of nitrite it is necessary to subtract the test value from a higher control value, with low activity levels producing very small differences between two much larger absorbance values. Thus, for low activity values, the difference in optical

density measured between the control and test assay lies within the limits of accurate measurement inherent in the assay. Indeed the average value for the absorbance difference at 540nm as a percentage of the control value is 3.35 per cent for M_3 plants (this value does not include Klaxon 48 which gives wild type control levels of activity), almost half the amount that can be measured accurately with this assay. Thus, the absolute amount of nitrite reductase activity expressed by these M_3 plants remains uncertain.

These inherent errors in measuring the amount of nitrite reduced should be unbiased, yet for all assays performed a positive value, albeit extremely low, was obtained. This suggests there was some form of bias on the assay allowing some low level loss of nitrite in the test compared to the control assays. Non-enzymic reduction of nitrite occurred in spinach and maize in dithionite-reduced methyl viologen nitrite reductase assays with boiled enzyme controls (Joy and Hageman, 1966). Attempts at analysis of non-enzymic loss of nitrite during *in vitro* methyl viologen nitrite reductase assays using leaf extract from minus-nitrate grown wild type barley plants were inconclusive. The average differences in the amount of nitrite lost between the test assay and the designed controls were small enough to lie within the limits of accuracy determined by the manipulations involved in measuring the amount of nitrite lost.

It is assumed that the diazotisation/coupling reaction used in the determination of nitrite is independent of the enzymic reaction involving dithionite-reduced methyl viologen when excess dithionite is removed by vigorous aeration. However, there is some evidence to suggest that dithionite may interfere with the determination of nitrite using sulphanilamide and NED. Dithionite undergoes at least three reactions prior to the diazotisation step. Dithionite reacts with the oxidised methyl viologen to form the radical cation and produces bisulphite (Danehy and Zubritsky, 1974). Dithionite also decomposes autocatalytically to produce bisulphite and thiosulphite (Danehy and Zubritsky, 1974) and when the assay is terminated by vigorous aeration the remaining dithionite is oxidised to bisulphite (Danehy and Zubritsky, 1974). Thus, prior to the determination of the nitrite concentration in the assay the dithionite has been converted to bisulphite and, to a lesser extent, thiosulphate. Senn *et al.*, (1976) showed that bisulphite and thiosulphite produce a significant decrease in the sensitivity of the diazotisation and coupling reactions with sulphanilamide and NED producing only 53 per cent and 69 per cent of the nitrite control values.

This interference in nitrite detection could be responsible for the apparent total-extract *in vitro* nitrite reductase activity observed in the M_2 selections lacking detectable

nitrite reductase cross reacting material. However, controls for *in vitro* methyl viologen nitrite reductase assays were altered to overcome this problem (section 4.2.5.4.), with controls performed exactly the same as the assays except that the tissue extract was withheld until after the termination of the assay by vortexing. It was noticeable that the control assay solutions (lacking tissue extract) turned from blue to clear almost immediately after the start of vortexing, whereas the test assays (containing tissue extract) took much longer to clear, requiring 20-25 seconds of vigorous vortexing. Whether this enzyme extract stabilisation was mediated through the dithionite or affected the reduced methyl viologen directly is unknown, but it is clear that some interaction occurs with some component of the tissue extract, perhaps producing non-enzymic loss of nitrite that may be responsible for the small differences in absorbance between control and test assays that leads to an apparent activity.

4.3.4. Nitrite accumulation screen selection pressure

The characterisation of the chlorophyll-containing nitrite accumulators (from both the bulk and spike-harvested populations) has resulted in the separation of the selected lines into two groups. The first group possesses significant levels of nitrite reductase activity and contains nitrite reductase protein. The progeny of these plants do not accumulate nitrite after *in vivo* or *in vitro* treatment with nitrate. Thus, these selected lines appear to lack any heritable biochemical deficiency for nitrite reduction and may represent false positives from the nitrite accumulation screen.

The second group of selections lacked nitrite reductase protein after treatment with nitrate. These lines also lacked significant levels of *in vitro* methyl viologen nitrite reductase activity and possessed levels of *in vitro* methyl viologen nitrate reductase activity comparable with NADH-nitrate reductase levels in leaf tissue extracts.

Other types of mutation that may have been expected to cause nitrite accumulation, such as defects in production and transfer of reducing power via photosystem I (most chlorophyll-deficient plants failed to accumulate nitrite), recognition of the chloroplast/plastid and processing of the precursor protein, or synthesis and insertion of prosthetic groups have proved elusive. While some of these expected mutations may have been lethal to the plant and therefore would not be selected, it is clear that some form of selection pressure is present within the screen.

The screen design will not allow the selection of certain types of mutation such as leaky mutants, where the plant is able to reduce the amount of nitrite produced by nitrate reductase and thus will not accumulate any nitrite. Mutations which cause an over-production of protein, which may include mutations to regulatory loci will also appear as wild type and would not be selected.

Selection Golden Promise 4169 V did not accumulate nitrite *in vivo* but lacked detectable nitrite reductase cross reacting material (Fig 4.1). This was the first plant to demonstrate the independence of *in vivo* nitrite accumulation from known biochemical deficiencies in nitrite reduction (a lack of nitrite reductase cross reacting material), which would be expected to cause nitrite accumulation within the plant tissue. Indeed Golden Promise 4169 V would not have been isolated if it were not for the fact that spike Golden Promise 4169 was identified as carrying other nitrite accumulating plants (Golden Promise 4169 I and II). Thus, it is possible that other plants within the screened populations lacked nitrite reductase cross reacting material and nitrite reductase activity but did not accumulate nitrite. These plants would have appeared as wild type in the screen and remained unidentified. Other types of mutations may also have remained unidentified due to their failure to cause *in vivo* nitrite accumulation, thus producing the selection pressure observed within the screen.

Interestingly the M_3 progeny of selections lacking nitrite reductase cross reacting material which inherit this defect and also lack significant *in vitro* nitrite reductase activity do not accumulate nitrite after *in vivo* treatment with nitrate and are thus similar to the M_2 selection Golden Promise 4169 V. These plants must fail to accumulate nitrite due to the fact that nitrite is either not produced or when nitrite is produced it is immediately reduced to ammonium ions (or converted to some other compound) by nitrite reductase or some, as yet unidentified, activity.

M_3 selections are capable of producing nitrite since they accumulate nitrate, express *in vitro* nitrate reductase activity and, in selection Golden Promise 2406 V at least, possess *in vivo* nitrate reductase activity after treatment with nitrate. Therefore, non-accumulation of nitrite must be due to its reduction to ammonium ions, or conversion to some other compound.

Since a reliable *in vivo* assay for nitrite reduction could not be established attempts were made to detect ammonium ions, the product of nitrite reduction, after treatment with

nitrate and methionine sulphoximine, an inhibitor of glutamine synthetase (Ronzio *et al.*, 1969). Unfortunately ammonium produced during the photorespiratory breakdown of glycine interfered with the measurement of ammonium ions due to nitrate assimilation, even after treatment in an atmosphere of 2 per cent oxygen and 350ppm carbon dioxide, known to inhibit photorespiration (Tolbert, 1981).

The use of $^{15}NO_3$ as a tracer demonstrated that progeny from selected lines Golden Promise 2406 and Tweed 3999 were capable of incorporating low levels of nitrate into leaf protein. Golden Promise 2406 possessed 10 per cent and Tweed 3999 possessed 23 per cent of the ^{15}N incorporated by wild type controls (Table 4.10).

However, it remains unclear whether the incorporation of $^{15}NO_3$ into protein in these selected plants is due to nitrate assimilation (ie by the action of nitrate and nitrite reductase). Microbial contamination of the plant roots may have resulted in the assimilation of nitrate into amino acids before uptake by the plants. The plants may contain very low levels of nitrite reductase protein that are either below the limit of detection by western blot analysis or are altered in some way to prevent recognition by nitrite reductase antiserum. Thus, the plants may possess sufficient nitrite reductase activity to prevent an accumulation of nitrite and allow low levels of nitrate assimilation. Alternatively the selected lines may contain some other activity that is able to metabolise nitrite preventing its accumulation and allowing low level incorporation into protein.

Chapter 5

Genetic analysis of selected lines

5.1. Introduction

5.1.1. Genetic analysis of nitrite reduction

Little information is available on the genetics of nitrite reduction. This is undoubtedly due to the fact that mutant analysis, carried out so successfully on the nitrate reduction step, has not been performed in higher plants due to the previous lack of whole-plant mutants.

This is unfortunate since the inheritance of nitrite reductase is of particular interest because nitrite reductase activity is localised within the chloroplast allowing the possibility that the gene or genes involved in the production of a functional nitrite reductase activity may be coded by nuclear or chloroplastic DNA.

5.1.2. Isozyme analysis

Inheritance studies of isozymes suggest that the nitrite reductase structural gene is nuclear encoded (Heath-Pagliuşo *et al.*, 1984). These workers found three isozymes for nitrite reductase associated with different ecotypes in wild oats using starch gel electrophoresis. The inheritance patterns showed that two of the wild oat isozymes were coded by a single Mendelian locus with two codominant alleles, while no variation was detected for the third isozyme.

However isozyme inheritance studies with inbred lines of diploid barley, hexaploid wheat and tetraploid durum wheat were not possible since no isozyme variation was found in any of the sixty nine lines tested (Heath-Pagliuso *et al.*, 1984). This may be due to the highly regulated nature of the enzyme, since Johnson (1976) suggested that highly regulated enzymes exhibit little electrophoretic variation unlike that observed for the "general housekeeping" enzymes that are highly polymorphic.

Nitrite reductase activities have been separated by ion exchange chromatography on DEAE-cellulose in several plant species (Kutscherra *et al.*, 1987). Three species (*Triticum aestivum*, *Galium aparine* and *Pisum sativum*) contained two isozymes (NiR_1 and NiR_2) in both leaf and root tissue while *Zea mays* contained two leaf isozymes but only one root enzyme (Kutscherra *et al.*, 1987). This is contrary to the work of Dalling *et al.*, (1973) who found two forms of nitrite reductase (NiR_1 and NiR_2) in root tissue as well as scutellum and etiolated shoots in *Zea mays*.

NiR_2 from non-chlorophyllous tissues (roots and scutellum) was found to resemble the green leaf enzyme in *Zea mays* (Dalling *et al.*, 1973) with similarities in isoelectric point, molecular weight, inhibition properties, pH stability, thermal stability, pH optima and Km values. NiR_1 and NiR_2 from leaf tissue also resembled one another with respect to pH optima, temperature optima, molecular weight and Km values (Kutscherra *et al.*, 1987). However significant NiR_1 activity was only expressed during the seed germination period and was not influenced by the supply of nitrate and light. During further plant development NiR_1 activity decreased leaving NiR_2 as the sole nitrite reductase activity which was found to depend strongly on the presence of exogenous nitrate (Kutscherra *et al.*, 1987).

Therefore, although isozymes for nitrite reductase exist in some higher plants at least, they appear to be either tissue specific or developmentally regulated. The studies of Dalling *et al.*, (1973) and Kutscherra *et al.*, (1987) were performed on single cultivars, thus the extent of isozyme variability within different cultivars representing the same species, which may be used for inheritance studies, remains unknown.

5.1.3. Mutant analysis

The isolation of whole plant mutants defective in nitrite reduction should allow genetic analysis of this step in the pathway. Cross pollination between selected plants, homozygous recessive for the defect, and wild type barley plants containing wild type alleles should produce F_1 progeny seed that are heterozygous for the defect. The ratios with which the F_2 progeny, derived from the self pollination of the F_1 generation plants, segregate to produce recessive homozygous mutants and wild type phenotype plants, both homozygous and heterozygous, should confirm the location of the genes responsible for the defect. Recovery of homozygous mutants:wild type in the Mendelian ratio of 1:3 would indicate that the genes responsible were nuclear coded (Fig 5.1.) whereas a chloroplastic location would only allow recovery of the mutants from the maternal line since all organelles are inherited from the maternal parent.

This approach requires that the selected plants grow to maturity and are fertile. However, M_2 bulk-harvested populations of barley mutagenised in the M_1 generation with sodium azide express negative side effects from the chemical mutagenic treatment, including slow growth, sterility and premature death which may be exascerbated by the accumulation of toxic levels of nitrite in the screen for nitrite reduction-deficient plants. These side effects

Fig 5.1. Expected segregation pattern within populations of F_2 progeny from F_1 plants derived from reciprocal crosses between selected nitrite accumulators and wild type plants.

Nuclear coded gene

Let N be the wild type allele (nitrite non-accumulator)

n be the mutant allele (nitrite accumulator)

Reciprocal crosses

NN x nn			nn x NN		
	n	n		N	N
N	Nn	Nn	n	nN	nN
N	Nn	Nn	n	nN	nN

F_1 seed genotype : Nn

phenotype : All nitrite non-accumulators

F_1 self pollinated

Nn x Nn			nN x nN		
	N	n		n	N
N	NN	Nn	n	nn	nN
n	nN	nn	N	Nn	NN

F_2 seed genotype : nn : Nn : NN 1 : 2 : 1

phenotype : accumulator : non-accumulator 1 : 3

resulted in the loss of many of the selected plants, and so the mutation they carry, before seed can be recovered through either self pollination or crosses with wild type plants.

In an attempt to overcome the loss of nitrite accumulating lines due to premature death of the selected plants the method of harvesting the mutagenised M_2 seed was altered. Bulk seed was no longer collected with a combine harvester, rather, M_1 spikes carrying the M_2 seed were collected manually. M_2 seed from each M_1 spike was then screened for nitrite accumulation.

The rationale behind this approach is that any spike selected for carrying a homozygous recessive mutant will have a high probability of carrying the mutation in the heterozygous form in one or more of the remaining seed carried on the spike. This is due to the process of producing homozygous mutants within M_2 populations derived from M_1 generations treated with sodium azide. The mutational event occurs within a single cell of the M_1 embryo treated with the mutagen producing a point mutation in an allele of interest (in this case those affecting nitrite reduction) on one of the homologous chromosomes thus producing a heterozygous cell for that particular allele.

By random chance the mutational event may occur within a cell which will ultimately give rise to those cells destined to undergo meiosis in the formation of gametes (ova and pollen). The haploid gametes produced by the meiotic division of a cell heterozygous for a particular allele will produce 2 cells with wild type alleles and 2 cells with mutated alleles.

The gametes containing either wild type or mutated alleles may then fuse during self pollination to form diploid M_2 embryos as part of a M_1 spike. The fusion of gametes will occur randomly allowing the formation of heterozygous and homozygous embryos. When the gametes both carrying the mutated allele fuse the resulting M_2 embryo will be homozygous and can be identified as a mutant by suitable screening (nitrite accumulation). However, if either the ova or pollen which fuse carry the mutated allele while the other gamete carries a wild type allele, the resulting M_2 embryo will be heterozygous for the mutation and will appear as a wild type plant. When both gametes contain a wild type allele, the resulting embryo will be a homozygous wild type.

Heterozygous embryos carried on the same spikes as homozygous mutants may be identified by segregation analysis of the M_3 progeny. The M_3 progeny of heterozygotes should segregate in the Mendelian ratio of 1:3 for nitrite accumulators:nitrite non-accumulators (Fig 5.2). In this way the homozygous mutation may be recovered

Fig 5.2. Expected segregation patterns for nitrite accumulation in populations of M_3 progeny derived from selected M_1 spikes.

Let N be the wild type allele (nitrite non-accumulator)

n be the mutant allele (nitrite accumulator)

Assume pollen or ova produced by selected M_1 spikes may contain either a wild type or mutant allele.

M_1 spike self pollination	n x n	n x N	N x n	N x N
M_2 seed	nn	nN	Nn	NN
	homozygous nitrite accumulator	heterozygous nitrite non-accumulator		homozygous nitrite non-accumulator

All self pollination of homozygotes will produce homozygotes

M_2 heterozygotes self pollination

nN x nN

	n	N
n	nn	nN
N	Nn	NN

Nn x Nn

	N	n
N	NN	Nn
n	nN	nn

M_3 seed nn : nN : NN 1 : 2 : 1

accumulator : non-accumulator 1 : 3

independently of whether the original selection survived. The mutation will also be maintained in the heterozygous form, since the nitrite non-accumulators will segregate 1:2 for wild type homozygotes:heterozygotes (Fig 5.2). Thus, the need for time consuming maintenance of the homozygous plants in the hydroponic growth system should be removed since the heterozygotes should grow on compost in the greenhouse with nitrate as a nitrogen source.

5.2. Results

5.2.1. Segregation within F_2 generations

F_1 seed was obtained from reciprocal cross pollinations between selected nitrite accumulators and wild type barley plants for 5 plants from the bulk-harvested screen and 2 plants from the spike-harvested screen (Table 5.1). The seed was grown under greenhouse conditions and allowed to self pollinate to produce F_2 seed.

5.2.1.1. *In vivo* nitrite accumulation

Since the majority of progeny plants derived from self pollination of selected nitrite accumulators failed to express the phenotype of *in vivo* nitrite accumulation (section 4.2.5.1.), the strategy for analysing the segregation patterns within F_2 generations by a simple screen for *in vivo* nitrite accumulation was no longer available. It was necessary, therefore, to test for the phenotype of a lack of leaf tissue nitrite reductase cross reacting material which was known to be inherited by the progeny from some of the selected plants (section 4.2.5.13.).

5.2.1.2. Nitrite reductase cross reacting material

F_2 progeny from 3 F_1 plants derived from cross pollinations between wild type (pollen recipient) plants and Golden Promise 2406 V (pollen donor plant) were screened for segregation by testing for the lack of detectable nitrite reductase cross reacting material, by western blotting, in leaf tissue after treatment with nitrate. A total of 69 F_2 seedlings, derived from 3 F_1 plants, were analysed with all plants producing detectable levels of nitrite reductase cross reacting material (Fig 5.3).

Thus, it appears that the loss of nitrite reductase cross reacting material in the M_2 nitrite accumulating selection Golden Promise 2406 V was not due to a mutation or, more likely, the F_1 progeny seed derived from cross pollinations between wild type (pollen recipient) plants and Golden Promise 2406 V (pollen donor plant) were the product of self pollination by the wild type plants. In this case the populations screened for segregation of a lack of nitrite reductase cross reacting material would comprise totally of wild type plants and would be expected to contain nitrite reductase cross reacting material.

Table 5.1. F_1 seed recovered from reciprocal crosses between selected nitrite accumulators and wild type barley plants.

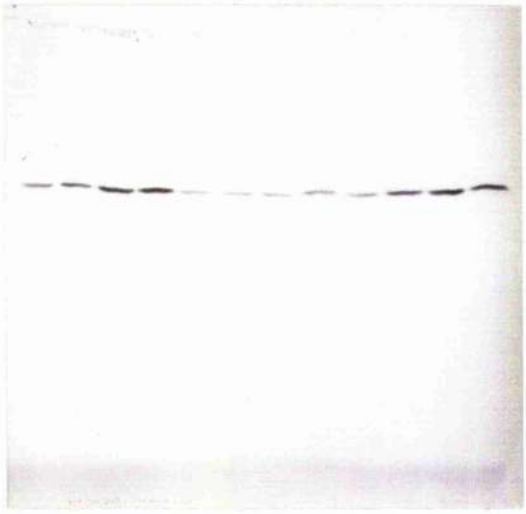
selection	F_1 seed collected from crosses	
	selection R x wt D	wt R x selection D
Golden Promise 10/3	2	8
Klaxon 33/4	16	66
Golden Promise 92/6	5	6
Patty 106/9	0	19
Golden Promise 138/12	3	0
Golden Promise 2406 V	0	4
Tweed 3999 II	4	19

Reciprocal crosses between selected nitrite accumulators and wild type barley plants were performed by hand. R : female parent (pollen recipient plant), D : male parent (pollen donor plant), wt : wild type.

Fig 5.3. Segregation analysis of nitrite reductase cross reacting material in populations of F_2 progeny from F_1 plants derived from cross pollinations between selection Golden Promise 2406 V and wild type barley plants.

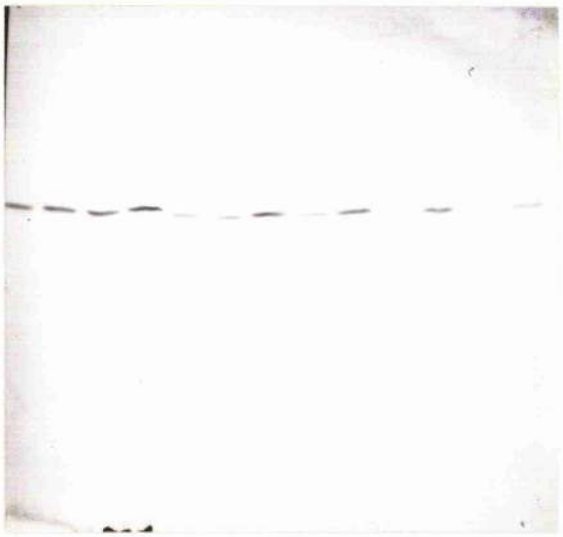
3 F_1 seed derived from cross pollinations between Golden Promise 2406 V (pollen donor plant) and Golden Promise wild type (pollen recipient plants) were grown in compost under greenhouse conditions and F_2 progeny seed collected. The F_2 generations were analysed for segregation of the phenotype of loss of detectable leaf nitrite reductase cross reacting material in 7 day old seedlings (4 days germination in the dark followed by 2 days growth in the light plus 24 hours treatment with 25mM KNO_3) by western blot analysis. All seedlings tested from the 3 different F_2 populations contained nitrite reductase cross reacting material (A,B and C).

A



B

C



5.2.1.3. *In vitro* nitrite accumulation

F_2 progeny from one F_1 plant, derived from a cross pollination between wild type (pollen recipient plant) and selection Tweed 3999 II (pollen donor plant) were screened for segregation by testing for *in vitro* nitrite accumulation. A total of 92 F_2 seedlings were screened with 27 seedlings giving a positive test for nitrite accumulation (Fig 5.4). The observed ratio is not significantly different from the Mendelian ratio of 1:3 nitrite accumulator:nitrite non-accumulator at the 10 per cent level of significance with a Chi-square value of 2.17 (Table 5.2). There was a 100 per cent correlation between *in vitro* nitrite accumulation and loss of nitrite reductase cross reacting material (Fig 5.4).

5.2.2. *In vivo* nitrite accumulation by F_2 selections

F_2 progeny of the F_1 plant, derived from cross pollination between Tweed 3999 and wild type, identified as *in vitro* nitrite accumulators were analysed for *in vivo* nitrite accumulation. The 14 day old seedlings were placed in individual pots of vermiculite and watered with half-strength Hoaglands nutrient solution containing 25mM KNO_3. After 24 hours leaf tissue was analysed for *in vivo* nitrite accumulation. Only 2 of the four F_2 seedlings tested accumulated nitrite *in vivo* (Table 5.3).

The same seedlings were then repotted with washed vermiculite and treated with nitrate-less half-strength Hoaglands nutrient solution for a further 24 hours and then leaf tissue was again analysed for *in vivo* nitrite accumulation. All the seedlings tested did not contain nitrite (Table 5.3) including those seedlings that had previously accumulated nitrite (Table 5.3). Thus, these seedlings have the capacity to metabolise or remove nitrite from leaf tissue.

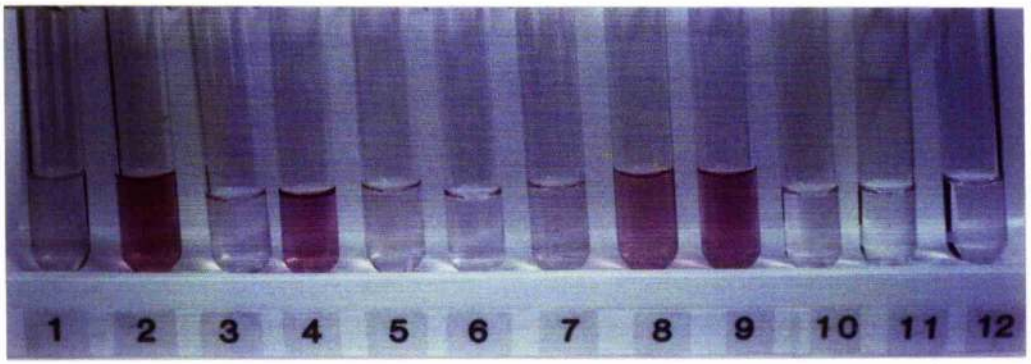
5.2.3. Identification of M_2 heterozygotes carried on selected M_1 spikes

A total of 175 M_2 seeds were carried on the 12 M_1 spikes selected for carrying one or more *in vivo* nitrite accumulators (Table 5.4). All the remaining M_2 seed carried on these selected spikes were grown and screened for nitrite accumulation. Any seedlings found accumulating nitrite were placed in the hydroponic growth system while non-accumulators were grown in pots of compost under greenhouse conditions. 84 nitrite non-accumulating M_2 plants were fertile (Table 5.4). These M_2 plants were allowed to self pollinate producing 84 M_3 families each derived from a single M_2 seed carried on selected M_1 spikes. These families

Fig 5.4. Segregation analysis of F_2 progeny of F_1 plant derived from cross pollinations between selection Tweed 3999 II and wild type barley plants by *in vitro* nitrite accumulation and loss of nitrite reductase cross reacting material.

One F_1 seed derived from cross pollination between Tweed 3999 II (pollen donor plant) and Tweed wild type (pollen recipient plant) was grown in compost under greenhouse conditions and F_2 progeny seed collected. The 7 day old F_2 seedlings (4 days germination in the dark followed by 2 days growth in the light watered with nitrate-less half-strength Hoaglands nutrient solution) were then analysed for segregation by *in vitro* nitrite accumulation (A). The leaf tissue was removed from the incubation solution and after micro-extraction was analysed for loss of nitrite reductase cross reacting material (B) by western blotting. There was 100 per cent correlation between *in vitro* nitrite accumulation and loss of nitrite reductase cross reacting material.

A



B

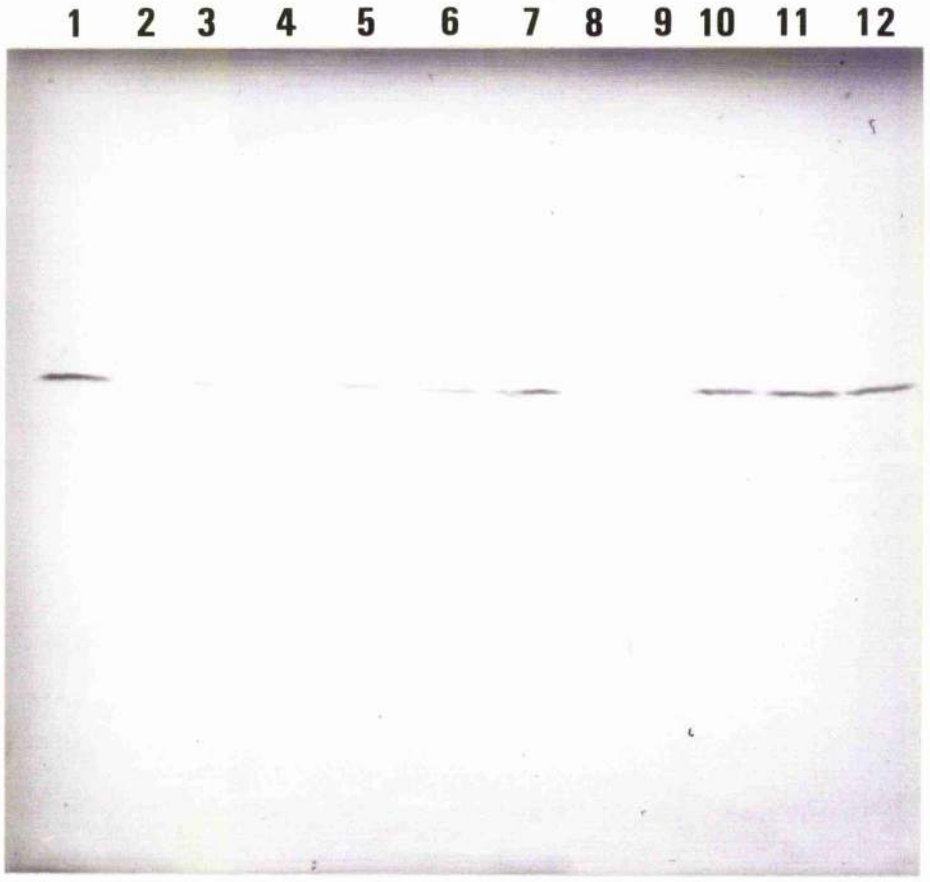


Table 5.2. Segregation of F_2 progeny for *in vitro* nitrite accumulation.

	number of plants		
phenotype	observed	expected	Chi-squared (1:3)
nitrite accumulator	27	23	2.17
nitrite non-accumulator	65	69	

F_2 seed from self pollination of F_1 plants derived from cross pollinations between Tweed wild type (pollen recipient) plants and selection Tweed 3999 II (pollen donor plant) were sown in individual positions in grids within trays of vermiculite and germinated in the dark for 6 days followed by 3 days growth in the light watered with half-strength Hoaglands nutrient solution. Each seedling was then analysed for leaf tissue *in vitro* nitrite accumulation.

Table 5.3. *In vivo* nitrite accumulation by F_2 selected plants.

Plant		*in vivo* nitrite accumulation μmoles nitrite/gm fresh weight		
		24	48	Hours
F_2	1	0.0	0.0	
	2	3.4	0.0	
	3	0.0	0.0	
	4	4.1	0.0	
wt	1	0.0	0.0	
	2	0.0	0.0	
	3	0.0	0.0	
	4	0.0	0.0	

14 day old fully green F_2 progeny of F_1 plant, derived from cross pollinations between Tweed 3999 and wild type plants, identified as *in vitro* nitrite accumulators were potted into individual pots of vermiculite and watered with half-strength Hoaglands nutrient solution containing 25mM KNO_3. After 24 hours the leaf tissue was analysed for *in vivo* nitrite accumulation and the plants repotted into washed vermiculite and watered with nitrate-less half-strength Hoaglands nutrient solution. After a further 24 hours the leaf tissue was again analysed for *in vivo* nitrite accumulation.

Table 5.4. Number of M_2 seed carried on selected M_1 spikes and M_3 seed produced from self pollination of M_2 nitrite non-accumulating plants.

	number				
spike	M_2 seed on M_1 spike	germinated	fertile	M_2 spikes	M_3 seed
Corniche 879	14	13	6	23	140
Doublet 603	11	6	0	0	0
Digger 679	11	8	1	7	89
Digger 860	15	119	8	87	11
Klaxon 48	9	2	1	2	4
Golden 2406 Promise	17	16	14	214	1,777
Golden 4169 Promise	14	12	6	47	335
Tweed 3999	23	19	15	114	972
Klaxon 1010	15	12	10	110	883
Klaxon 2760	20	19	15	144	1,596
Natasha 1489	12	5	1	13	89
Natasha 2114	14	13	6	59	356

The remaining M_2 seed carried on selected spikes were grown in pots of vermiculite and tested for *in vivo* nitrite accumulation. Those seedlings that accumulated nitrite were placed in the hydroponic growth system while those that did not accumulate nitrite were grown in pots of compost in the greenhouse and allowed to self pollinate producing M_3 seed carried on M_2 spikes. The M_3 seed was then harvested to form families which were then tested separately for the presence of homozygous segregants.

contained a total of 6,952 M_3 seed (Table 5.4) some of which may be homozygous for those mutations responsible for nitrite accumulation by the original selections.

5.2.3.1. M_3 family *in vivo* nitrite accumulation

Families of M_3 seed derived from 31 M_2 nitrite non-accumulating plants carried on 5 selected M_1 spikes (Corniche 879, Digger 678, Digger 860, Golden Promise 2406 and Golden Promise 4169) were screened for *in vivo* nitrite accumulation (Tables 5.5 and 5.6). There was no segregation for the phenotype of leaf tissue *in vivo* nitrite accumulation after treatment with nitrate, with every seedling screened giving a negative test for nitrite accumulation.

5.2.3.2. M_3 family *in vitro* nitrite accumulation

11 M_3 families derived from M_2 nitrite non-accumulating plants carried on selected M_1 spike Golden Promise 2406 were screened for *in vitro* nitrite accumulation. There was no segregation for the phenotype of leaf tissue *in vitro* nitrite accumulation (Table 5.7) with every seedling giving a negative result for nitrite accumulation.

5.2.3.3. Chlorophyll deficiency

3 M_1 spikes (Corniche 879, Digger 679 and Digger 860) were identified as carrying M_2 chlorophyll-deficient nitrite accumulators (section 3.2.3.2.). The M_3 progeny derived from the self pollination of normal chlorophyll-containing M_2 members of these three families were grown under nitrite accumulation screening conditions. The plants were then visually checked for chlorophyll deficiency and screened for *in vivo* nitrite accumulation after treatment with nitrate Table 5.6).

Three M_3 families derived from one M_1 spike (Corniche 879) produced chlorophyll-deficient seedlings (Table 5.6). The observed segregation ratios for chlorophyll-deficiency were not significantly different, at the 25 per cent level of confidence, from the Mendelian ratio of 1:3, chlorophyll-deficient:chlorophyll-containing, for each of the three families (Corniche 879 I, III and VI) with Chi-square values of 0.18, 0.58 and 0.33 respectively (Table 5.8). One other family derived from spike Corniche 879 and all families derived from spikes Digger 679 and Digger 860 produced only normal chlorophyll-containing green seedlings (Table 5.6).

Table 5.5. Segregation analysis of M_3 families derived from selected M_1 spikes Golden Promise 2406 and Golden Promise 4169 by *in vivo* nitrite accumulation.

Family code	M_3 seed sown	number seedlings germinated	nitrite accumulators
GP 2406 I	38	16	0
GP 2406 II	38	13	0
GP 2406 III	38	19	0
GP 2406 IV	50	27	0
GP 2406 VI	50	33	0
GP 2406 VII	50	34	0
GP2406 VIII	28	8	0
GP2406 IX	28	11	0
GP2406 X	28	2	0
GP2406 XI	28	13	0
GP2406 XII	28	9	0
GP4169 III	12	4	0
GP4169 VIII	12	10	0
GP4169 X	12	2	0
GP4169 XI	12	3	0
GP4169 XIII	12	4	0
GP4169 XIV	12	7	0

M_2 nitrite non-accumulating seedlings carried on selected M_1 spikes were grown in compost under greenhouse conditions and allowed to self pollinate to produce M_3 families. Seed from each family was grown under nitrite accumulation screening conditions (4 days germination in the dark followed by 2 days growth in the light plus 16-20 hours treatment with 25mM KNO_3) and then analysed for segregation of the leaf tissue *in vivo* nitrite accumulating phenotype.

Table 5.6. Segregation amongst M_3 plants derived from selected M_1 spikes for chlorophyll deficiency and nitrite accumulation.

M_3 family		seed sown	number chlorophyll normal	chlorophyll deficient	nitrite accumulators
CO 879	I	18	10	3	0
	III	39	9	2	0
	VI	32	8	4	0
	VII	14	0	0	0
DR 679	II	56	39	0	0
DR 860	III	70	45	0	0
	IV	56	16	0	0
	VI	54	38	0	0
	VII	55	37	0	0
	VIII	70	30	0	0
	IX	53	17	0	0
	X	70	25	0	0
	XII	48	6	0	0
	XIV	56	23	0	0

Self pollinated M_3 seed from families derived from M_1 selected spikes that produced chlorophyll-deficient nitrite accumulators were grown under nitrite accumulation screen conditions (4 days germination in the dark followed by 2 days growth in the light watered with half-strength Hoaglands nutrient solution). The seedlings were visually screened for chlorophyll deficiency and then after 24 hours treatment with half-strength Hoaglands containing 25mM KNO_3 were screened for *in vivo* nitrite accumulation to determine whether any of the original M_2 seed carried on the selected M_1 spike were heterozygous for the mutation responsible for either or both of the observed phenotypes of chlorophyll deficiency and nitrite accumulation. CO : cv. Corniche, DR : cv. Digger.

Table 5.7. Segregation of M_3 families derived from M_1 spike Golden Promise 2406 by *in vitro* nitrite accumulation.

Family	M_3 seed sown	number seedlings germinated	nitrite accumulators
GP 2406 I	56	21	0
GP 2406 III	56	10	0
GP 2406 IV	51	22	0
GP 2406 VI	56	11	0
GP 2406 VII	30	8	0
GP 2406 VIII	15	0	0
GP 2406 IX	56	11	0
GP 2406 X	21	16	0
GP 2406 XII	56	30	0
GP 2406 XIV	56	7	0
GP 2406 XV	48	14	0

Leaf tissue (about 10 mg) from fully green 14 day old seedlings watered with half-strength Hoaglands nutrient solution was incubated in 1ml 0.1M KNO_3 in the light for 16-20 hours. The tissue was removed and the incubation solution analysed for nitrite by the addition of 1ml 1% sulphanilamide in 3N HCl and 1ml 0.02% NED.

Table 5.8. Segregation of chlorophyll-deficient M_3 seedlings derived from self pollination of M_2 chlorophyll-containing selections carried on M_1 spikes.

Family	chlorophyll deficient	chlorophyll normal	Chi-squared (1:3)
Corniche 879 I	3 (3.25)	10 (9.75)	0.18
Corniche 879 III	2 (2.75)	9 (8.25)	0.58
Corniche 879 VI	4 (3.00)	8 (9.00)	0.33

M_3 seed, derived from self pollination of chlorophyll-containing nitrite non-accumulating M_2 plants carried on M_1 spikes selected for carrying chlorophyll-deficient nitrite accumulating individuals, were germinated in the dark for 4 days followed by 2 days growth in the light watered with half-strength Hoaglands nutrient solution. The seedlings were then visually checked for chlorophyll deficiency. Numbers in parenthesis denote the expected frequencies to give a 1:3 ratio.

5.3. Discussion

5.3.1. Production of F_2 populations

Reciprocal cross pollinations were performed more successfully for the plants isolated by the bulk-harvested screen than for plants isolated from spike-harvested screening. Crosses were performed with only 2 plants selected from the spike screen (Golden Promise 2406 V and Tweed 3999 II) which produced F_1 seed. Cross pollinations were complicated by the poor fertility of the selected plants as seen in the low number of M_3 seed produced by allowing the selected plants to self pollinate (section 3.2.2.). The morphology of the flowering spikes was also altered, with the spikes being smaller and more fragile under the manipulations necessary for performing the cross pollinations. This made identification of the correct developmental stages for pollen donation and acceptance difficult, resulting in unsuccessful pollinations.

Cross pollinations between M_2 nitrite accumulators selected from the bulk-harvested screen and wild type plants were more successful, with F_1 seed produced from crosses performed with the 5 fertile selected plants (Table 5.1). The M_2 bulk-harvested selections grew more vigorously in the hydroponic growth system than those selected from the spike-harvested screen and the flowering spike development and morphology were more typical of wild type barley. The bulk harvested M_2 selections were more fertile than those plants selected from the spike harvested screen producing almost 5 times as much M_3 seed with a doubling of the seed set/spike (8.4 compared to 4.4 seed/spike, section 3.2.2.). Thus, the manipulations required for cross pollinations were easier to perform.

5.3.2. Segregation within F_2 populations

The inability of the M_3 progeny, derived from self pollination of the M_2 bulk-harvested selections to accumulate nitrite, after either *in vivo* or *in vitro* treatment with nitrate, made analysis of the segregation of homozygotes (if any mutation existed) within the F_2 populations difficult. No biochemical data is available on the M_2 selections from the bulk-harvested screen since the techniques required to analyse these plants without compromising their survival were not established at that time. Therefore, although the M_3 progeny, derived from self pollination of the M_2 selections, express lower levels of *in vitro* nitrite reductase activity and nitrite reductase cross reacting material in 7 day old seedlings (section 4.2.4.3. and 4.2.4.7.) it is not known whether these traits are inherited from the parents or are a

physiological phenomenon. Thus, there is no certain basis on which any homozygous mutants within segregating populations could be selected.

The M_3 progeny derived from the self pollination of M_2 plants selected from the spike screen also failed to accumulate nitrite after *in vivo* treatment with nitrate. However, biochemical analysis of M_2 spike-harvested plants allowed the identification of distinct, heritable biochemical differences compared to wild type barley plants. The M_3 progeny lacked detectable nitrite reductase cross reacting material and expressed negligible levels of *in vitro* methyl viologen nitrite reductase activity as well as expressing *in vitro* methyl viologen nitrate reductase activity in crude tissue extracts. Leaf tissue from selected lines also gave a positive reaction *for in vitro* nitrite accumulation (section 4.2.5.2.). These heritable differences were used as the basis for a screening procedure to analyse segregation patterns within the F_2 generation.

The F_2 population derived from the F_1 generation produced by cross pollination between Tweed wild type (pollen recipient) plants and selection Tweed 3999 II (pollen donor plant) segregated after *in vitro* nitrite accumulation analysis (Fig 5.4). In a Chi-square test the ratio between *in vitro* nitrite accumulators and nitrite non-accumulators was not significantly different from the Mendelian ratio of 1:3 at the 10 per cent level of confidence. The same F_2 population was also screened for the loss of nitrite reductase cross reacting material by western blotting and exhibited a 100 per cent correlation between *in vitro* nitrite accumulation and loss of nitrite reductase protein (Fig 5.4). Thus, the mutation responsible for the loss of nitrite reductase protein resulting in *in vitro* nitrite accumulation occurs within a single nuclear recessive locus in selection Tweed 3999 II.

The analysis of 3 F_2 populations, derived from crosses between selection Golden Promise 2406 V and wild type, for leaf nitrite reductase cross reacting material demonstrated that all plants tested contained detectable levels of nitrite reductase cross reacting material (Fig 5.3). However, it is likely that the F_1 seed was the product of self pollination by the wild type female parent and not a true cross between Golden Promise 2406 V and wild type. Thus, the F_2 populations screened would have been wild type and so no segregation for the loss of nitrite reductase cross reacting material would be expected.

5.3.3. Segregation within M_3 families

Heterozygotes amongst families of M_2 seed carried on selected M_1 spikes have yet to be found for the phenotype of nitrite accumulation. All families tested failed to segregate after *in vivo* nitrite accumulation analysis (Tables 5.5 and 5.6). This may be due either to the lack of heterozygotes amongst the families tested or segregating homozygous mutants do not express the phenotype of *in vivo* nitrite accumulation in the same manner that self pollinated M_3 progeny failed to accumulate nitrite after *in vivo* treatment with nitrate (section 4.2.5.1.).

Other phenotypes inherited by the progeny of selected plants (a lack of nitrite reductase cross reacting material, *in vitro* nitrite accumulation or, in some cases, chlorophyll deficiency) allowed segregation analysis amongst M_3 populations from families derived from selected M_1 spikes. Segregation analysis of M_3 families derived from spike Golden Promise 2406 by *in vitro* nitrite accumulation failed to produce any segregant nitrite accumulators (Table 5.7). Thus, none of the families derived from M_1 spike Golden Promise 2406 analysed for *in vitro* nitrite accumulation originated from a heterozygous M_2 seed. Spike Golden Promise 2406 may not have carried any heterozygous M_2 seed, or any heterozygote present may have been one of the 5 M_2 seed carried by spike Golden Promise 2406 that failed to produce M_3 progeny due either to non-germination of the M_2 seed or sterility of the M_2 plant.

However, 53 families, containing over 4,200 M_3 seed, from the remaining 6 selected M_1 spikes producing chlorophyll-containing nitrite accumulators remain to be screened by *in vitro* nitrite accumulation (Table 5.4). Some of these families may be derived from heterozygous parent plants and segregate to produce homozygous *in vitro* nitrite accumulators.

5.3.4. Segregation of chlorophyll-deficient M_3 seedlings

Recovery of chlorophyll-deficient seedlings in the M_3 progeny of phenotypically normal M_2 plants carried on selected M_1 spikes (Table 5.5) demonstrates the general application of the spike harvesting and screening strategy. The isolation of M_1 spikes carrying homozygous mutants allowed the subsequent identification of M_2 heterozygous individuals carried on the same M_1 spikes, after segregation of their progeny for chlorophyll deficiency. The overall segregation ratio within the three families for chlorophyll-deficient:normal seedlings of 1:3 indicates that a mutation within a single recessive nuclear

gene is responsible for the chlorophyll-deficient phenotype displayed by these plants. It is unknown whether the nitrite accumulation and chlorophyll deficiency within the M_2 selections were due to one or more different mutations within the same plant since none of the chlorophyll-deficient seedlings accumulated nitrite *in vivo* after treatment with nitrate.

The lack of chlorophyll-deficient plants in any of the 9 M_3 families derived from spike Digger 860 demonstrates that production of M_2 heterozygotes carried by M_1 spikes is by no means certain. This is due to the random manner in which the original M_1 cells carrying the heterozygous mutation and wild type cells interact to form the M_2 embryos (Fig 5.2). Thus, it is necessary to screen families derived from as many spikes as possible to increase the probability of recovering M_3 homozygous plants. Recovery of homozygotes is also affected by the germination and fertility of the M_2 plants carried on the M_1 spike which may reduce the number of families available for screening. Spike Digger 679 produced only 1 M_3 family from 9 M_2 seeds (Table 5.6) which considerably reduces the probability of recovering heterozygotes, if they were originally present.

5.3.5. Allelic analysis

It had been hoped to use cross pollinations between the selected plants to investigate allelism. However, this proved difficult since the selected plants expressed poor seed setting when selfed and were difficult to manipulate in cross pollinations, as described above. More importantly they matured and flowered at different times due to their erratic growth rates (section 3.2.2.) and isolation at different times. M_3 progeny also expressed abnormal growth rates when maintained in the hydroponic growth system making allelic testing between M_3 generation plants derived from the various selections difficult.

Chapter 6

Environmental control of nitrite reductase

6.1. Introduction

6.1.1. Environmental influences on nitrite reductase

Nitrate and light influence the development of nitrite reductase activity. Whether nitrate , or nitrite produced as a result of nitrate reduction is the true inducer of nitrite reductase is unclear. Nitrite induces nitrite reductase activity in *Lemna minor* when present at 5mM, but nitrite reductase activity decreases at higher nitrite concentrations followed by plant death within a few days, (Joy 1969b). Unlike *Lemna minor* where only nitrite reductase activity was induced by nitrite, both nitrite reductase and nitrate reductase activities increased in non-photosynthetic tobacco XD cell cultures exposed to nitrite (Chroboczek Kelker and Filner, 1971). However, nitrite was found to be toxic to XD cells above 1mM (Chroboczek Kelker and Filner, 1971), placing some doubt upon the *in vivo* role of nitrite as inducer for nitrite reductase. Use of the molybdenum analogue tungstate, a specific inhibitor of development of nitrate reductase activity (Wray and Filner, 1970), and hence of nitrite synthesis, did not inhibit development of nitrite reductase activity in response to nitrate (Chroboczek Kelker and Filner, 1971), suggesting that nitrate does not have to be reduced to nitrite to cause an increase in nitrite reductase activity. This is further supported by reports that barley mutants defective in nitrate reduction, and so unable to synthesise nitrite, possessed levels of nitrite reductase activity that were comparable with those found in wild type control plants (Warner *et al.*, 1977; Bright *et al.*, 1983; Steven *et al.*, 1986). The nitrate content of these nitrate reductase-deficient mutants were also comparable with nitrate levels found in wild type control plants (Warner *et al.*, 1977; Steven, 1986). Thus, in barley, it appears that nitrate is capable of increasing nitrite reductase activity directly, and not via its reduction to nitrite.

The interaction between nitrate and light in the regulation of nitrite reductase levels has been examined by several workers. In the absence of both light and nitrate, nitrite reductase activity is low in mustard cotyledons (Rajasekhar and Mohr, 1986a), pea leaves (Gupta and Beevers, 1983), and rice leaves (Ogawa and Ida, 1987), while high levels of nitrite reductase activity are found in plants treated with both light and nitrate (Rajasekhar and Mohr, 1986a; Gupta and Beevers, 1983; Ogawa and Ida, 1987). In pea, nitrate addition has no effect on nitrite reductase activity in dark-grown plants (Gupta and Beevers, 1983), whereas nitrite reductase activity in dark-grown rice seedlings (Ogawa and Ida, 1987), or mustard (Rajasekhar and Mohr, 1986a) exposed to nitrate is about 50 per cent of the level

found in light-grown plants. Light alone has no effect on the development of nitrite reductase activity in mustard, (Schuster *et al.*, 1987), suggesting that the light effect cannot express itself in the absence of nitrate and that nitrate is the true "inducer" of nitrite reductase activity.

Red/far red light pulse experiments in maize (Sharma and Sopory, 1984) and mustard (Rajasekhar and Mohr, 1986a) indicate the involvement of phytochrome in the light regulation of nitrite reductase. Red light treatment given up to 8 hours before nitrate addition can strongly enhance subsequent nitrate induction of nitrite reductase activity (Sharma and Sopory, 1984). The red light effect is reversed by far red light, but photoreversibility is reduced to zero within 2 hours. Sharma and Sopory argue that phytochrome induces a "biochemical signal" within 2 hours which can persist and cause enhancement of nitrate induction of nitrite reductase activity for a period of 8-12 hours. Signal storage is proposed to be a means of enabling the plant to maintain the appropriate levels of nitrite reductase (and indeed also of nitrate reductase) during the dark period of the natural light/dark cycle (Schuster *et al.*, 1987).

Since photo-oxidative damage of the chloroplasts abolishes the action of nitrate and light on nitrite reductase activity development in mustard cotyledons, Rajasekhar and Mohr (1986a) have postulated an indirect role for chlorophyll via plastid development to produce a "plastidic signal". This signal is a prerequisite for the induction of nitrite reductase by nitrate and light. However, nitrate and light (phytochrome) act independently of each other in controlling the appearance of nitrite reductase activity. Nitrate is the inducer proper, whereas phytochrome modulates the extent of the nitrate induced response.

Increases in nitrite reductase activity are correlated with increases in nitrite reductase cross reacting material in wheat (Small and Gray, 1984), pea (Gupta and Beevers, 1984) and rice (Ogawa and Ida, 1987). These results indicate that the environmentally induced modulation of extractable nitrite reductase activity involves alteration of the enzyme level by *de novo* synthesis and is not mediated by a reversible activation-inactivation of the existing enzyme.

Poly(A)$^+$ RNA from nitrate-treated light-grown wheat (Small and Gray, 1984) and pea (Gupta and Beevers, 1987) supports the synthesis of nitrite reductase whilst that isolated from nitrate-deficient, dark-grown plants does not. These results suggest that nitrate and light modulate the synthesis of nitrite reductase mRNA. Evidence that nitrate at least acts in

this manner is provided by the use of the cloned spinach nitrite reductase cDNA gene in Northern blot analysis. Application of nitrate to ammonium/light-grown plants leads to a substantial increase in nitrite reductase specific mRNA (Back *et al.*, 1988). While this increase in nitrite reductase mRNA in response to nitrate may be due to an increase in the rate of transcription of the nitrite reductase structural gene, other possibilities, such as altered RNA processing or decreased mRNA degradation still exist.

Little information is available on end-product control of nitrite reductase levels in higher plants. In *Lemna minor* (Joy, 1969a) and tobacco XD cell cultures (Chroboczek Kelker and Filner, 1971), ammonium and casein hydrolysate, respectively, inhibit development of nitrite reductase activity in response to nitrate. Rajasekhar and Mohr, (1986b) demonstrated that in dark-grown mustard cotyledons ammonium, given together with nitrate, strongly inhibits the nitrate induced development of nitrite reductase.

In nearly all studies performed on the regulation of nitrite reductase no information is presented detailing the tissue nitrate content in plants grown under various environmental conditions. This is a major omission, since it is clear that nitrate is required for the induction of nitrite reductase activity (Rajasekhar and Mohr, 1986a). Therefore, the possibility exists that some of the observed differences in development of nitrite reductase activity reported in the literature may be due to effects on the internal level of nitrate.

6.1.2. Root nitrite reductase

Very little is known of the regulation of nitrite reductase activity in root tissue. The enzyme is present at very low or undetectable levels in plastids isolated from intact pea roots grown on distilled water (Emes and Fowler, 1983). Nitrite reductase activity shows a marked increase of about 10-fold in plastids isolated from intact pea roots grown with nitrate (Emes and Fowler, 1983). However, the molecular basis for the observed increase in nitrite reductase activity is unknown.

6.2. Results

6.2.1. Nitrite reductase polyacrylamide gel electrophoresis

Barley possesses a single functional nitrite reductase activity (dithionite-reduced methyl viologen as electron donor) in both leaf and root tissue from light-grown plants exposed to nitrate. The methyl viologen nitrite reductase activities from both tissues comigrate during non-denaturing polyacrylamide gel electrophoresis (Fig 6.1).

Nitrite reductase cross reacting material is recognised by antibodies raised against purified barley leaf nitrite reductase (Ip *et al.*, 1987) in both leaf and root tissue from light-grown plants exposed to nitrate (Fig 6.2) after western blot analysis. Nitrite reductase cross reacting material from both leaf and root comigrate with authentic purified leaf nitrite reductase during denaturing SDS polyacrylamide gel electrophoresis (Fig 6.2).

6.2.2. Effect of nitrate

In the absence of nitrate *in vitro* methyl viologen nitrite reductase activity is present at low levels in both leaf and root tissues (0.4-0.6μmoles nitrite reduced/mg protein/hour) of light-grown plants. Leaf tissue from plants treated with nitrate in the light show an almost 16-fold increase in activity (7.9 μmoles nitrite reduced/mg protein/hour, Fig 6.3) and a 10-fold increase in root tissue (4.75μmoles nitrite reduced/mg protein/hour, Fig 6.4).

Nitrite reductase cross reacting material is present at low levels in leaf tissue from plants grown without nitrate in the light (Fig 6.3), whereas root tissue nitrite reductase cross reacting material is virtually undetectable (Fig 6.4). Plants treated with nitrate in the light show an increase in nitrite reductase cross reacting material in both leaf and root tissues (Fig 6.3 and 6.4).

Plants grown in the absence of nitrate have undetectable levels of tissue nitrate in leaf and root (Fig 6.3 and 6.4). Nitrate accumulation occurs in both leaf (10.8μmoles nitrate/gm fresh weight) and root tissue (13.1μmoles nitrate/gm fresh weight) over the following 28 hour period after treatment of plants with nitrate in the light (Fig 6.3 and 6.4).

In the absence of nitrate leaf tissue *in vitro* nitrate reductase activity was undetectable (Table 6.1) but increased in tissue from plants treated with nitrate in the light (from 0 to 264 nmoles nitrite produced/mg protein/hour) over the the following 28 hour period.

Fig 6.1. Non-denaturing polyacrylamide gel electrophoresis and gel staining for nitrite reductase activity in barley leaf and root tissue extract.

Leaf and root tissue extract from nitrate and light-grown plants were subjected to non-denaturing polyacrylamide gel electrophoresis followed by gel staining for methyl viologen nitrite reductase activity.
1: wild type leaf extract, 2: wild type root extract. Both lanes were loaded with 100μg protein.

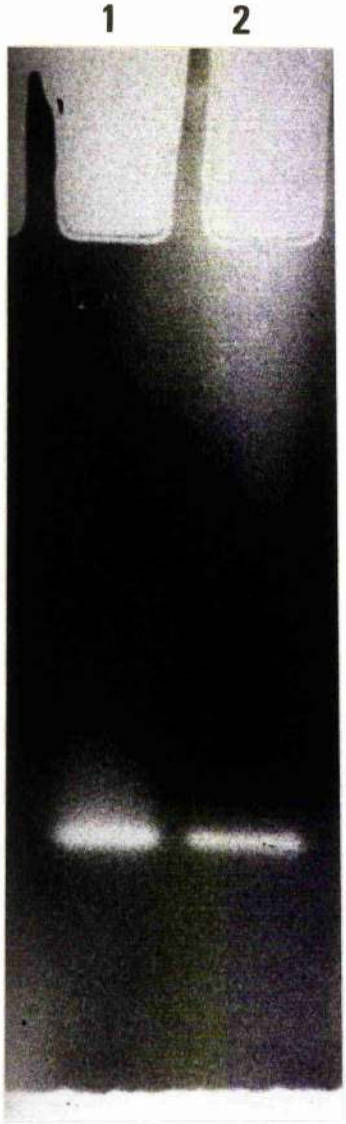

1
2

Fig 6.2. Nitrite reductase cross reacting material in barley leaf and root tissue.

Leaf and root tissue extract from nitrate and light-grown barley plants were subjected to SDS polyacrylamide gel electrophoresis followed by western blotting. Nitrite reductase cross reacting material was analysed with nitrite reductase antiserum raised against purified barley leaf nitrite reductase.

1: purified barley leaf nitrite reductase (1μg protein), 2: leaf tissue extract (100μg protein), 3: root tissue extract (100μg protein).

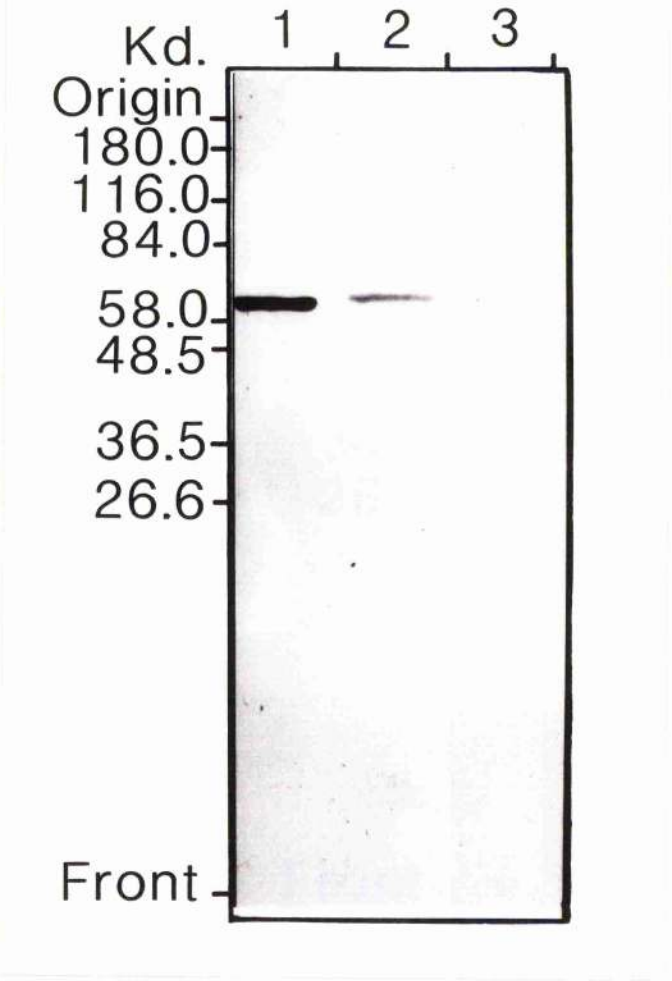

Kd.
1
2
3
Origin
180.0
116.0
84.0
58.0
48.5
36.5
26.6
Front

Fig 6.3. The effect of nitrate on the development of leaf nitrite reductase and tissue nitrate content.

Fully green 6 day old barley seedlings (4 days germination in the dark followed by 2 days growth in the light, watered with half-strength Hoaglands nutrient solution minus nitrate) were treated in the light with either half-strength Hoaglands nutrient solution minus nitrate (●), or treated with half-strength Hoaglands plus 25mM KNO_3 (○). Samples of leaf tissue were analysed for nitrite reductase cross reacting material by western blotting. Lanes 1-6: 0,4,8,12,24 and 28 hours after treatment with 25mM KNO_3, lanes 7-12: 0,4,8,12,24 and 28 hours after treatment with nitrate-less half Hoaglands nutrient solution. The samples were also assayed for *in vitro* methyl viologen nitrite reductase activity and leaf nitrate content. Each analysis was performed in duplicate and the experiment repeated once.

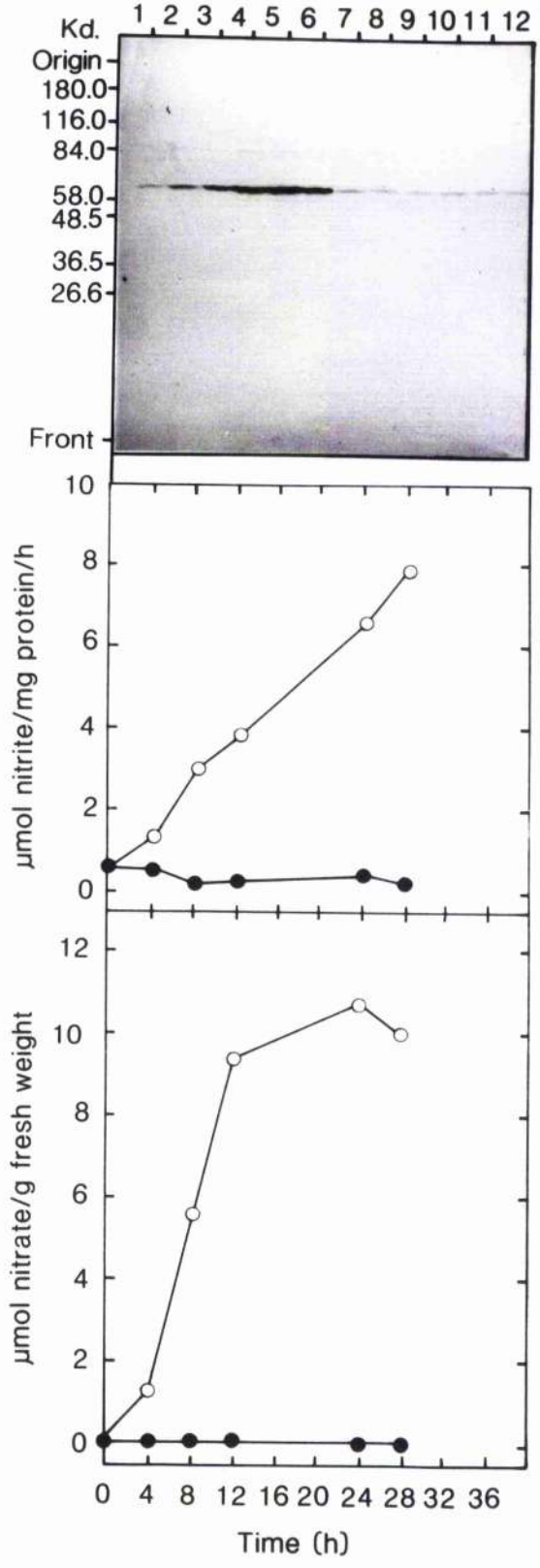

1 2 3 4 5 6 7 8 9 10 11 12
Kd.
Origin
180.0
116.0
84.0
58.0
48.5
36.5
26.6
Front
µmol nitrite/mg protein/h
µmol nitrate/g fresh weight
0 4 8 12 16 20 24 28 32 36
Time (h)

Fig 6.4. The effect of nitrate on the development of root nitrite reductase and tissue nitrate content.

Fully green 6 day old barley seedlings (4 days germination in the dark followed by 2 days growth in the light, watered with half-strength Hoaglands nutrient solution minus nitrate) were treated in the light with either half-strength Hoaglands nutrient solution minus nitrate (●), or treated with half-strength Hoaglands plus 25mM KNO_3 (○). Samples of root tissue were analysed for nitrite reductase cross reacting material by western blotting. Lanes 1-6: 0,4,8,12,24 and 28 hours after treatment with 25mM KNO_3, lanes 7-12: 0,4,8,12,24 and 28 hours after treatment with nitrate-less half Hoaglands nutrient solution. The samples were also assayed for *in vitro* methyl viologen nitrite reductase activity and leaf nitrate content. Each analysis was performed in duplicate and the experiment repeated once.

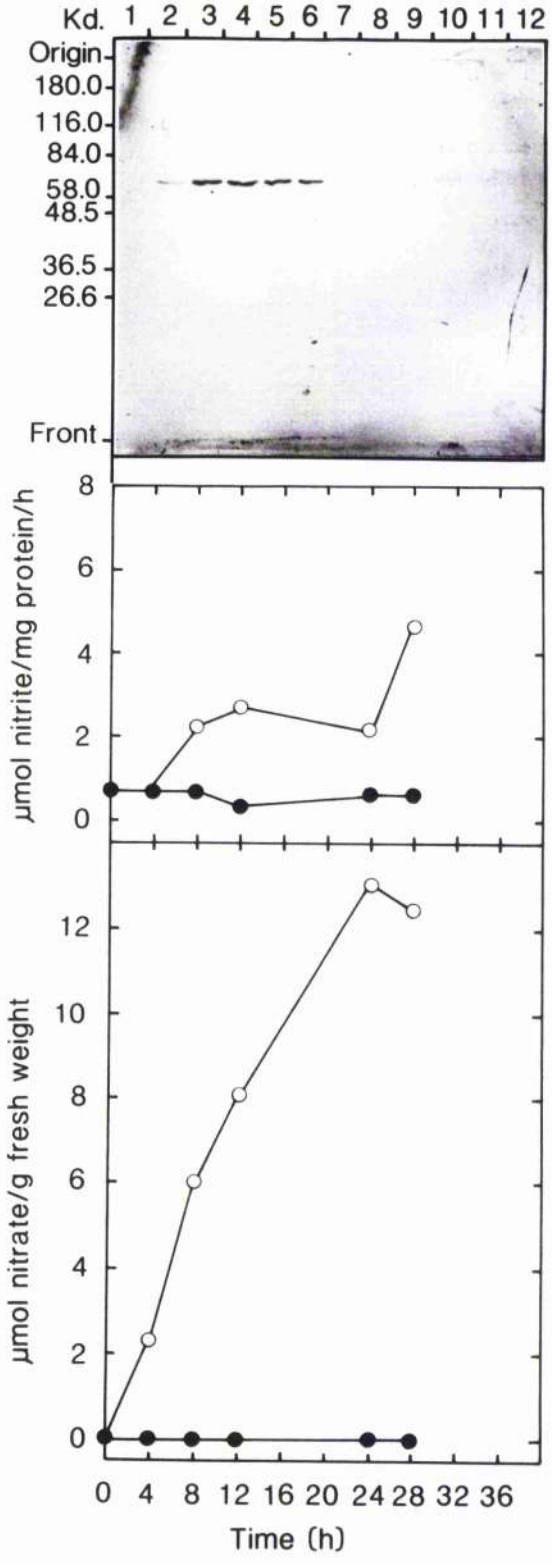

Kd.
1 2 3 4 5 6 7 8 9 10 11 12
Origin
180.0
116.0
84.0
58.0
48.5
36.5
26.6
Front
µmol nitrite/mg protein/h
0
2
4
6
8
µmol nitrate/g fresh weight
0
2
4
6
8
10
12
0 4 8 12 16 20 24 28 32 36
Time (h)

Table 6.1. ***In vitro*** **NADH-nitrate reductase activity from 7 day old barley leaf tissue under various growth conditions with light, dark, nitrate, glutamine and ammonium ions.**

Growth conditions	nitrate reductase activity nmoles nitrite produced/mg protein/hour						
	0	4	8	12	24	28	hours
- nitrate + light	0	0	0	0	0	0	
+ nitrate + light	0	28	79	117	191	264	
+ nitrate - light	0	10	26	45	28	N.D.	
+ nitrate + ammonium	0	8	25	87	160	170	
+ nitrate + glutamine	0	19	45	85	145	150	

N.D. : not determined

Fully green 6 day old barley seedlings (germinated in the dark for 4 days followed by 2 days growth in the light) were treated with 25mM KNO_3 and maintained in the light or transferred to darkness, or were treated with 25mM KNO_3 and either 25mM NH_4Cl or 25mM glutamine and maintained in the light. Samples of leaf tissue were removed and assayed for *in vitro* NADH-nitrate reductase activity. Each analysis was performed in duplicate and each experiment repeated once.

6.2.3. Effect of light

In the absence of light leaf tissue *in vitro* methyl viologen nitrite reductase activity remains at low levels in plants treated with nitrate (0.5-1.1μmoles nitrite reduced/mg protein/hour, Fig 6.5) compared to an almost 13-fold increase in activity in leaf tissue from plants exposed to nitrate plus light (from 0.5 to 6.4μmoles nitrite reduced/mg protein/hour, Fig 6.5) over 24 hours.

In vitro methyl viologen nitrite reductase activity in root tissue increases 7 to 10-fold in plants exposed to nitrate in the light (from 0.5 to 3.6μmoles nitrite reduced/mg protein/hour, Fig 6.6) and in the dark (from 0.5 to 5.0μmoles nitrite reduced/mg protein/hour, Fig 6.6).

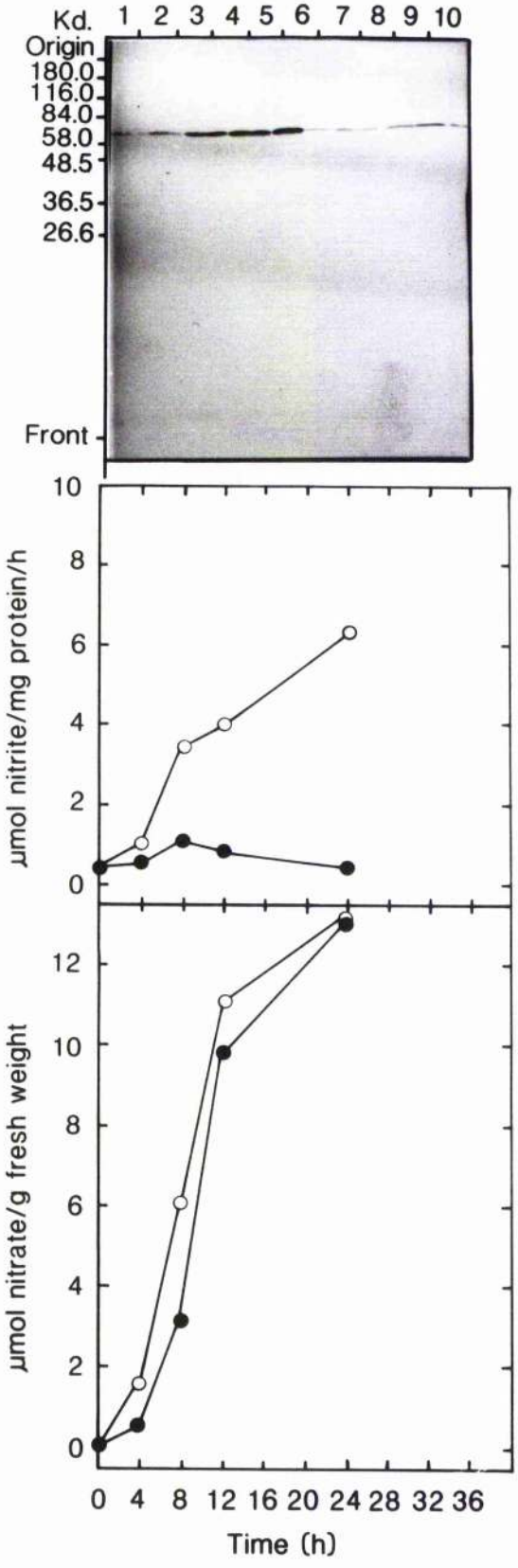
Nitrite reductase cross reacting material remains at low levels in leaves of plants exposed to nitrate in the dark (Fig 6.5). However, root nitrite reductase cross reacting material increases in plants exposed to nitrate in both the dark and the light (Fig 6.6).

Nitrate accumulation occurs in leaf tissue (13.2μmoles nitrate/gm fresh weight) whether the plants were treated with nitrate in the light or dark, (Fig 6.5). Similarly, root tissue accumulates nitrate (13.1-13.6μmoles nitrate/gm fresh weight) to the same level in plants treated with nitrate in the light and dark (Fig 6.6).

Leaf tissue from plants treated with nitrate in the dark shows a slight increase in *in vitro* nitrate reductase activity (from 0 to 45 nmoles nitrite produced/mg protein/hour) which appears to peak after 12 hours and is on average 30 per cent of the activity found in leaf tissue from plants treated with nitrate in the light (Table 6.1).

Fig 6.5. Development of leaf nitrite reductase and tissue nitrate content in the light.

Fully green 6 day old barley seedlings (4 days germination in the dark followed by 2 days growth in the light, watered with half-strength Hoaglands nutrient solution minus nitrate) were treated with half-strength Hoaglands nutrient solution plus 25mM KNO_3 and maintained either in the light (○), or transferred to the dark (●). Samples of leaf tissue were analysed for nitrite reductase cross reacting material by western blotting. Lanes 1-5: 0,4,8,12 and 24 hours after treatment with 25mM KNO_3 in the light, lanes 6-10: 0,4,8,12 and 24 hours after treatment with 25mM KNO_3 in the dark. The samples were also assayed for *in vitro* methyl viologen nitrite reductase activity and leaf nitrate content. Each analysis was performed in duplicate and the experiment repeated once.


Kd.
1 2 3 4 5 6 7 8 9 10
Origin
180.0
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26.6
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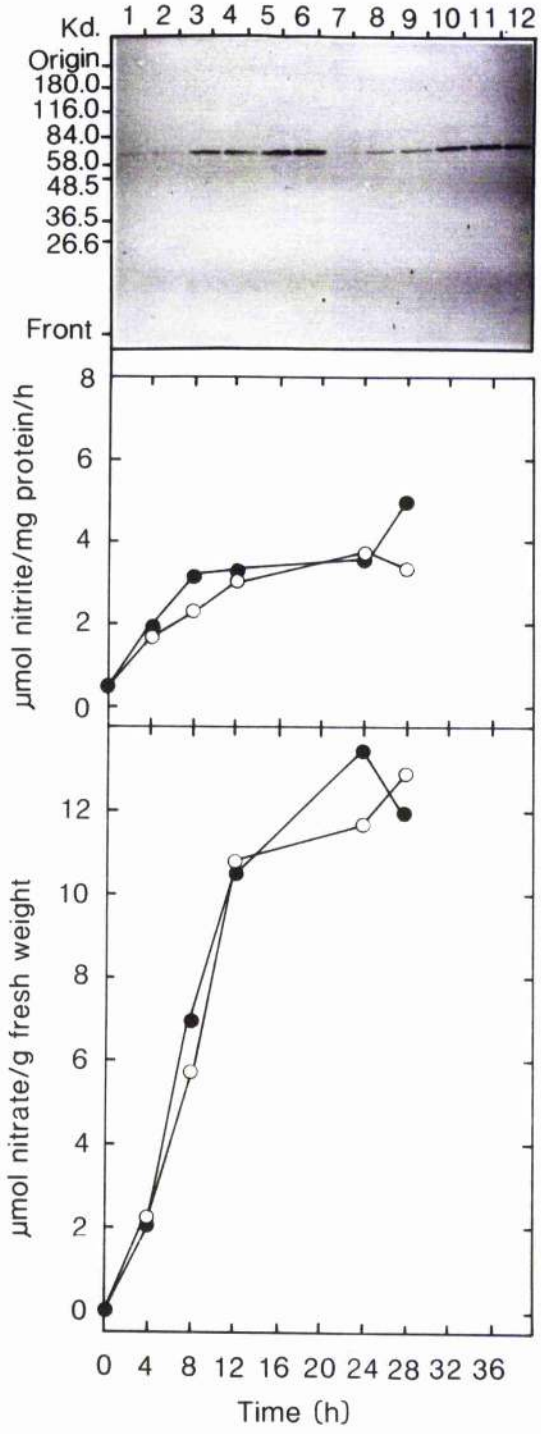
μmol nitrite/mg protein/h
0 2 4 6 8 10
μmol nitrate/g fresh weight
0 2 4 6 8 10 12
0 4 8 12 16 20 24 28 32 36
Time (h)

Fig 6.6. Development of root nitrite reductase and tissue nitrate content in the light.

Fully green 6 day old barley seedlings (4 days germination in the dark followed by 2 days growth in the light, watered with half-strength Hoaglands nutrient solution minus nitrate) were treated with half-strength Hoaglands nutrient solution plus 25mM KNO_3 and maintained either in the light (○), or transferred to the dark (●). Samples of root tissue were analysed for nitrite reductase cross reacting material by western blotting. Lanes 1-6: 0,4,8,12,24 and 28 hours after treatment with 25mM KNO_3 in the light, lanes 7-12: 0,4,8,12,24 and 28 hours after treatment with 25mM KNO_3 in the dark. The samples were also assayed for *in vitro* methyl viologen nitrite reductase activity and leaf nitrate content. Each analysis was performed in duplicate and the experiment repeated once.


Kd.
1 2 3 4 5 6 7 8 9 10 11 12
Origin
180.0
116.0
84.0
58.0
48.5
36.5
26.6
Front
μmol nitrite/mg protein/h
0
2
4
6
8
μmol nitrate/g fresh weight
0
2
4
6
8
10
12
0 4 8 12 16 20 24 28 32 36
Time (h)

6.2.4. Effect of end products

In vitro methyl viologen nitrite reductase activity increased almost 7-fold (from 0.7 to 4.7μmoles nitrite reduced/mg protein/hour, Fig 6.7) in leaf tissue from plants exposed to equimolar amounts of nitrate and ammonium ions. The activity is comparable with that found in leaf tissue from plants exposed solely to nitrate where nitrite reductase activity increased 10-fold (0.7-7.0 μmoles nitrite reduced/mg protein/hour, Fig 6.7).

Plants exposed to equimolar amounts of nitrate and glutamine exhibited an increase in leaf *in vitro* methyl viologen nitrite reductase activity of almost 6-fold (from 0.5 to 2.9 μmoles nitrite reduced/mg protein/hour, Fig 6.8). This increase in activity was just under 40 per cent of that measured in leaf tissue from plants treated with nitrate alone (from 0.5 to 7.4 μmoles nitrite reduced/mg protein/hour, Fig 6.8).

Leaf tissue from plants treated with equimolar amounts of either ammonium ions and nitrate or glutamine and nitrate showed very little difference in nitrite cross reacting material content compared to nitrate-grown controls (Fig 6.7 and 6.8).

Ammonium ions and glutamine produce a slight stimulation of *in vitro* methyl viologen nitrite reductase activity in assays performed with leaf tissue extracts from 7 day old nitrate-grown wild type seedlings. *In vitro* nitrite reductase activity was increased by an average of 26 per cent of the control value when either NH_4Cl or glutamine was exogenously added to the assay solution (Table 6.2). Increasing the final concentration of NH_4Cl or glutamine (from 10 to 30mM) in the assay had no further effect on the measured increase in *in vitro* nitrite reductase activity (Table 6.2).

The increase in nitrite reductase activity appears to be a non-specific effect since exogenously added NaCl or glycine also produced a stimulation of *in vitro* nitrite reductase activity (12 and 22 per cent of the control value respectively, Table 6.2).

Leaf tissue nitrate accumulation was similar in plants grown with nitrate (8.3μmoles nitrate/gm fresh weight) or nitrate plus ammonium ions (9.9 μmoles nitrate/gm fresh weight, Fig 6.7). Plants treated with nitrate or nitrate plus glutamine also accumulated nitrate to the same extent (13.4 μmoles nitrate/gm fresh weight, Fig 6.8).

Leaf *in vitro* nitrate reductase activity was lower in plants treated with nitrate plus ammonium ions (about 60 per cent activity) compared with plants treated with nitrate alone

Fig 6.7. Effect of ammonium ions on the development of leaf nitrite reductase and leaf tissue nitrate content.

Fully green 6 day old barley seedlings (4 days germination in the dark followed by 2 days growth in the light, watered with half-strength Hoaglands nutrient solution minus nitrate) were treated in the light with half-strength Hoaglands nutrient solution plus 25mM KNO_3 (●), or 25mM KNO_3 plus 25mM NH_4Cl (○). Samples of leaf tissue were analysed for nitrite reductase cross reacting material by western blotting. Lanes 1-6: 0,4,8,12,24 and 28 hours after treatment with 25mM, lanes 7-12: 0,4,8,12,24 and 28 hours after treatment with 25mM KNO_3 KNO_3 plus 25mM NH_4Cl. The samples were also assayed for *in vitro* methyl viologen nitrite reductase activity and leaf nitrate content. Each analysis was performed in duplicate and the experiment repeated once.

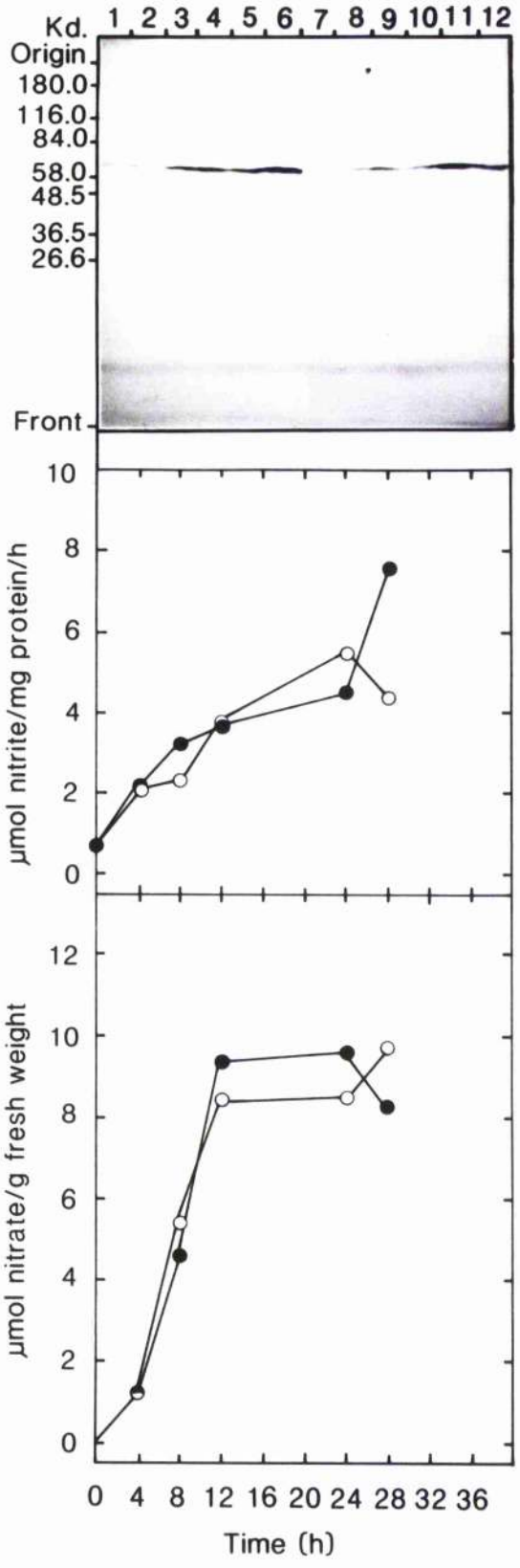
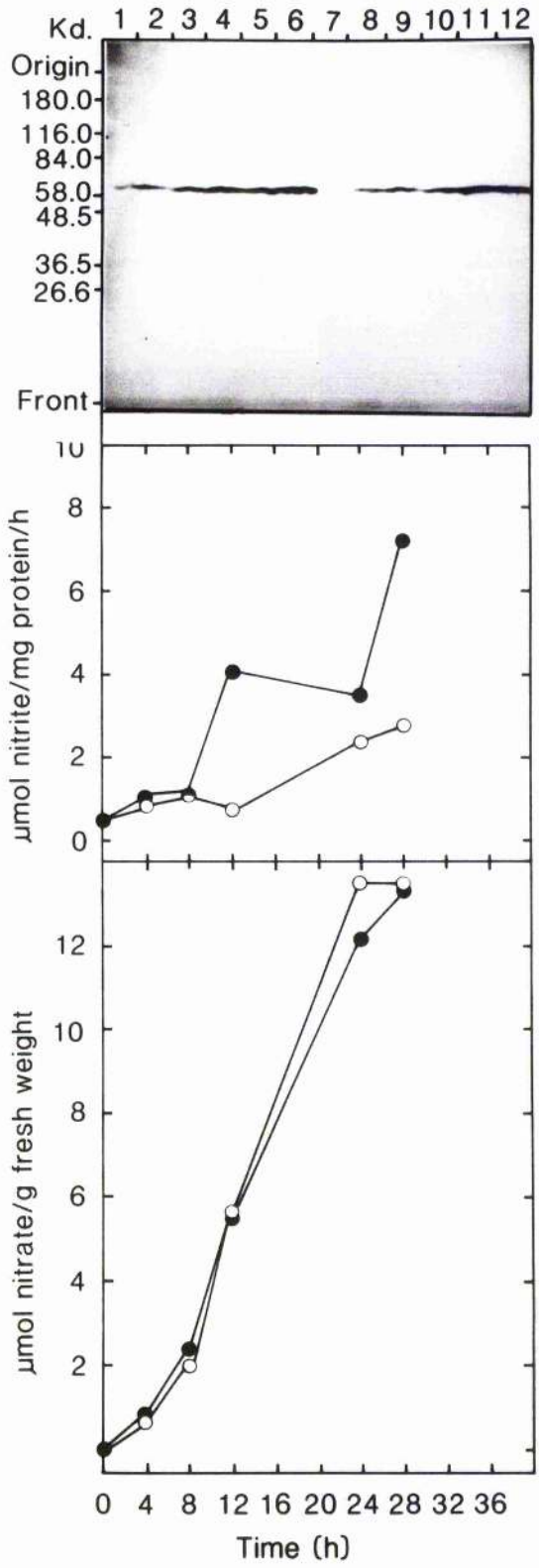

Kd.
1 2 3 4 5 6 7 8 9 10 11 12
Origin
180.0
116.0
84.0
58.0
48.5
36.5
26.6
Front
µmol nitrite/mg protein/h
0 2 4 6 8 10
µmol nitrate/g fresh weight
0 2 4 6 8 10 12
0 4 8 12 16 20 24 28 32 36
Time (h)

Fig 6.8. Effect of glutamine on the development of leaf nitrite reductase and leaf tissue nitrate content.

Fully green 6 day old barley seedlings (4 days germination in the dark followed by 2 days growth in the light, watered with half-strength Hoaglands nutrient solution minus nitrate) were treated in the light with half-strength Hoaglands nutrient solution plus 25mM KNO_3 (●), or 25mM KNO_3 plus 25mM glutamine (○). Samples of leaf tissue were analysed for nitrite reductase cross reacting material by western blotting. Lanes 1-6: 0,4,8,12,24 and 28 hours after treatment with 25mM KNO_3, lanes 7-12: 0,4,8,12,24 and 28 hours after treatment with 25mM KNO_3 plus 25mM glutamine. The samples were also assayed for *in vitro* methyl viologen nitrite reductase activity and leaf nitrate content. Each analysis was performed in duplicate and the experiment repeated once.


Kd.
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Origin
180.0
116.0
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26.6
Front
μmol nitrite/mg protein/h
0 2 4 6 8 10
μmol nitrate/g fresh weight
0 2 4 6 8 10 12
0 4 8 12 16 20 24 28 32 36
Time (h)

Table 6.2. *In vitro* nitrite reductase activity of wild type leaf tissue extract assayed in the presence of exogenously added ammonium ions or glutamine.

	in vitro nitrite reductase activity µmoles nitrite reduced/gm fresh weight/hour				
	0	10	20	30	(mM)
Glutamine	105	134	139	123	
Glycine	105	134	134	117	
NH_4Cl	105	137	130	133	
NaCl	105	121	114	117	

Leaf tissue from 7 day old (4 days germination in the dark followed by 2 days growth in the light and treated with 25mM KNO_3 for 24 hours) fully green seedlings was extracted and assayed for *in vitro* methyl viologen nitrite reductase activity in the presence of exogenously added ammonium ions or glutamine. Ammonium chloride or glutamine was added to the assay solutions to a final concentration of 0, 10, 20 and 30mM. Control assays were performed with exogenously added sodium chloride and glycine. Each assay was performed in duplicate and the experiment repeated once.

(Table 6.1). Plants treated with glutamine and nitrate also possessed lower levels of leaf *in vitro* nitrate reductase activity (about 66 per cent) compared to plants treated with nitrate alone (Table 6.1).

Ammonium ions and glutamine produce an inhibition of *in vitro* NADH-nitrate reductase activity in assays performed with leaf tissue extracts from 7 day old nitrate-grown wild type seedlings. *In vitro* nitrate reductase activity was on average 69 and 86 per cent of the control value when either ammonium ions or glutamine, respectively, was exogenously added to the assay solution (Table 6.3). Increasing the final concentration of glutamine in the assay had little further effect upon the decrease in activity (Table 6.3). However, a 3-fold increase in the final concentration of NH_4Cl (from 10 to 30mM) produced a decrease in nitrate reductase activity from 641 to 330 nmoles nitrate produced/gm fresh weight/hour (Table 6.3).

The decrease in nitrate reductase activity appears to be a non-specific effect since exogenously added NaCl or glycine also produced a decrease in *in vitro* nitrate reductase activity (80 and 64 per cent of the control value respectively, Table 6.3).

Table 6.3. *In vitro* nitrate reductase activity of wild type leaf tissue extract assayed in the presence of exogenously added ammonium ions or glutamine.

	in vitro nitrate reductase activity nmoles nitrite produced/gm fresh weight/hour				
	0	10	20	30	(mM)
Glutamine	731	641	701	541	
Glycine	731	420	491	500	
NH_4Cl	731	641	541	330	
NaCl	731	701	541	521	

Leaf tissue from 7 day old (4 days germination in the dark followed by 2 days growth in the light and treated with 25mM KNO_3 for 24 hours) fully green seedlings was extracted and assayed for *in vitro* NADH-nitrate reductase activity in the presence of exogenously added ammonium ions or glutamine. Ammonium chloride or glutamine was added to the assay solutions to a final concentration of 0, 10, 20 and 30mM. Control assays were performed with exogenously added sodium chloride and glycine. Each assay was performed in duplicate and the experiment repeated once.

6.3. Discussion

6.3.1. leaf and root tissue nitrite reductase

Nitrite reductase from barley leaf and root tissue possess the same molecular weight (Fig 6.2), mass:charge ratio (Fig 6.1) and have antigenic sites in common (Fig 6.2). Nitrite reductase from pea roots was also found to be of similar molecular weight compared to the leaf enzyme (Bowsher *et al.*, 1988). Nitrite reductase from leaf and root tissue from bean (Ishiyama *et al.*, 1985) and pea (Bowsher *et al.*, 1988) gave reactions of identity after Ouchterlony double diffusion experiments. This suggests that, in these species at least, the leaf and root activities are properties of the same or closely related protein species.

6.3.2. Influence of nitrate and light

Increases in *in vitro* methyl viologen nitrite reductase activity in response to nitrate and light are due to *de novo* synthesis of enzyme molecules in both leaf and root tissue, with concomitant increases in nitrite reductase activity and nitrite reductase cross reacting material (Fig 6.3) and not the result of some form of inactivation/activation mechanism of pre-existing enzyme molecules. Thus, regulation of nitrite reductase in barley leaf and root tissue in response to nitrate in the light is similar to that reported in leaf tissue of wheat (Small and Gray, 1984) and pea (Gupta and Beevers, 1984) where increases in nitrite reductase activity correlated with increases in nitrite reductase cross reacting material.

Leaf tissue requires the presence of both light and nitrate for full expression of nitrite reductase activity and nitrite reductase cross reacting material (Fig 6.5). Plants given nitrate in the dark were firstly exposed to light for 48 hours to allow normal maturation of chloroplasts and development of chlorophyll. Thus, the requirement for any "plastidic signal" due to chlorophyll development postulated by Rajasekhar and Mohr, (1986a) would be fulfilled. However, *in vitro* methyl viologen nitrite reductase activity and nitrite reductase cross reacting material remained essentially at a constant level throughout the 24 hours following exposure to nitrate in the dark. Thus, no light signal storage, of the type described by Schuster *et al.*, (1987) for mustard cotyledons, occurs in barley to allow the enhancement of nitrate induction of nitrite reductase in the dark, during the natural light/dark cycle.

The lack of such a system to regulate nitrite reductase activity levels is unlikely to be detrimental to the plant, since nitrite reductase is a relatively stable enzyme *in vivo*, with nitrite reductase activity reduced by only 28 per cent in nitrate-grown plants where nitrate

was subsequently withheld for 3 days (Gupta and Beevers, 1983). Therefore, the amount of nitrite reductase protein within the leaf tissue is likely to remain at an adequate level to reduce any nitrite, produced by nitrate reductase, to ammonium ions during the normal growth cycle of the plant.

In vitro NADH-nitrate reductase activity was undetectable in leaf tissue from light-grown plants in the absence of nitrate (Table 6.1). Somers *et al.*, (1983) demonstrated that the increase in nitrate reductase activity seen after nitrate treatment of barley plants is due to the *de novo* synthesis of nitrate reductase molecules.

Plants exposed to nitrate in the dark show an increase in leaf *in vitro* NADH-nitrate reductase activity which peaks 12 hours after exposure to nitrate (Table 6.1). The increases in activity are 30-40 per cent of those expressed in plants exposed to nitrate in the light (Table 6.1). Interestingly the development of *in vitro* NADH-nitrate reductase activity peaks after 12 hours suggesting that the lower level of induction by nitrate in the dark may be due to some form of cryptic light signal (Schuster *et al.*, 1987) to allow the tissue to respond to nitrate levels during the natural light/dark cycle.

Nitrate reductase is a relatively unstable enzyme and is turned over more rapidly than nitrite reductase. In pea extractable nitrate reductase activity in nitrate-grown plants that subsequently had nitrate withheld was reduced by 88 per cent within 3 days, compared to 28 per cent for extractable nitrite reductase activity (Gupta and Beevers, 1983). Thus, nitrate reductase activity levels are more susceptible to environmental changes, compared to nitrite reductase which is more stable *in vivo* and present at low levels even in the absence of nitrate. Therefore, a mechanism to allow development of nitrate reductase activity in response to nitrate in low light intensities or the natural dark period would presumably allow continuity and co-ordination of nitrate assimilation under environmental growth conditions.

The inability to synthesise nitrite reductase molecules in leaves of plants treated with nitrate in the dark is not due to the exclusion of nitrate (Fig 6.5). Nitrate accumulation by leaf and root tissue is unaffected by any of the environmental growth conditions tested in both leaf and root tissue (Figs 6.3-6.8). Therefore, regulation of nitrite reductase (and nitrate reductase) under the environmental conditions tested is not mediated by the control of nitrate availability to root and leaf tissue.

Light has no effect upon development of *in vitro* methyl viologen nitrite reductase activity and nitrite reductase cross reacting material in root tissue from nitrate-grown plants

(Fig 6.6). Nitrite reductase activity, due to *de novo* synthesis of enzyme molecules, increases to the same extent in roots from plants exposed to nitrate in the light or dark. Roots are excluded from light under natural growth conditions and are unable to absorb light. It should be expected, therefore, that light has no effect upon the regulation of the enzyme in root tissue.

6.3.4. Influence of ammonium ions and glutamine

Ammonium ions when given with nitrate do not affect leaf tissue *in vitro* nitrite reductase activity or nitrite reductase cross reacting material (Fig 6.7). However, *in vitro* nitrate reductase activity is reduced by about 40 per cent in leaf tissue extracts from plants treated with equimolar amounts of nitrate and ammonium ions compared to nitrate-treated control plants (Table 6.1). Both *in vitro* nitrate reductase and nitrite reductase activities are lower in leaf tissue extracts of plants grown with nitrate and glutamine compared to nitrate-treated control plants (Table 6.1 and Fig 6.8).

It is unclear whether the lower levels of leaf *in vitro* nitrite reductase activity observed in plants treated with glutamine and nitrate are due to changes in the level of nitrite reductase cross reacting material or some form of inactivation of nitrite reductase by glutamine. The levels of nitrite reductase cross reacting material appear to be the same in leaf tissue extracts from plants treated with nitrate and glutamine compared to nitrate-treated controls (Fig 6.8). However, exogenously added glutamine stimulates *in vitro* nitrite reductase activity (Table 6.2). Therefore, some form of inactivation of nitrite reductase molecules, by increases in the leaf tissue content of glutamine, at least, appears unlikely.

6.3.5. Accurate measurement of low levels of nitrite reductase activity

The measurement of low levels of *in vitro* nitrite reductase activity is difficult since the loss of nitrite between the control and test assay is less than the minimum error of measurement for the assay (5.8 per cent (section 4.2.5.4.)). These errors arising from the precision of the manipulations necessary to perform the assay should be unbiased, so that the measured value of nitrite lost should represent the actual nitrite reductase activity.

However, the nitrite reductase assays were performed with controls where dithionite was omitted and 0.95M sodium bicarbonate only was added. There is some evidence that dithionite and its derivatives are capable of interfering with the determination of nitrite content with sulphanilamide and NED (Danehy and Zubritsky, 1974; Senn *et al.*, 1976,

(section 4.3.3.)). This would result in an overstatement of the loss of nitrite by the test assay which contained dithionite. Therefore, it is uncertain whether the low levels of measured activity in minus-nitrate grown or dark-grown plants represents a true measure of the activity present, due to the low levels of nitrite reductase protein detectable in these plants, or is an overstatement of the activity, caused by an inherent bias from the design of the controls used.

6.3.6. Environmental control of nitrate assimilation

Although nitrite reductase is influenced by environmental factors such as nitrate availability, light intensity and the abundance of the end products of nitrate assimilation it is clear that nitrate reductase is the logical point to effect regulation of input of reduced nitrogen as it is the first and normally rate limiting enzyme in the assimilation of nitrate into amino-N (Beevers and Hageman, 1980).

Nitrate reductase activity is high in plants grown on nitrate in the light (Beevers and Hageman, 1969: Gupta and Beevers, 1983). The increases observed in nitrate reductase activity after nitrate treatment is due to *de novo* synthesis of nitrate reductase molecules (Somers *et al.*, 1983). Thus, both nitrate reductase and nitrite reductase exhibit the same form of regulation in response to nitrate and light, by controlling the amount of enzyme molecules synthesised within the plant tissues. This co-ordinated regulation clearly conserves the plant resources of amino-N when nitrate availability is low by preventing the futile production of nitrate and nitrite reductase protein molecules.

The reduced levels of leaf nitrate and nitrite reductase activity in plants treated with equimolar amounts of nitrate and either ammonium ions or glutamine may occur through either the control of the *de novo* synthesis of enzyme molecules or through some form of inactivation of existing enzyme molecules. Since both nitrate and the end products of nitrate assimilation are available to these plants it may be that the observed decrease in the level of nitrate and nitrite reductase activities constitute some form of fine control to balance the plants requirements for further nitrate assimilation and prevent over production of ammonium ions and/or glutamine.

Chapter 7

Immunolocalisation of nitrite reductase

7.1. Introduction

7.1.1. *In vivo* location of nitrite reductase

Nitrite reductase is widely accepted to be located within the chloroplasts in higher plant leaf tissue with fractionation studies generally demonstrating the coincidental sedimentation of nitrite reductase with chloroplast marker enzymes and chlorophyll (Ritenour *et al.*, 1967; Dalling *et al.*, 1972a; Miflin, 1974). Physiological studies also support the chloroplastic location of nitrite reductase with intact chloroplasts from a variety of species capable of photoreduction of nitrite to ammonium ions (Paneque *et al.*, 1963) and thence to α-NH_2 nitrogen (Anderson and Done, 1978) with reduced ferredoxin as the physiological electron donor.

More recently molecular studies have demonstrated that *in vitro* translation of $polyA^+$ RNA and immuno-precipitation of products with specific nitrite reductase antiserum reveals the synthesis of a peptide of higher molecular weight in both wheat (Small and Gray, 1984) and pea (Gupta and Beevers, 1985). In pea, this *in vitro* synthesised peptide can be cleaved, in a two-step process, to a peptide of the same size as that of the native enzyme by a proteinaceous extract from chloroplasts (Gupta and Beevers, 1987), suggesting that nitrite reductase mRNA is translated in the cytoplasm as a larger precursor protein which is transported into the chloroplast where the modified protein is functional *in vivo*. The existence of a precursor form of the protein has been confirmed by the molecular cloning of spinach nitrite reductase cDNA species (Back *et al.*, 1988). These workers showed that the precursor protein for nitrite reductase, with a molecular weight of about 66,000, has a 32 amino acid extension at the N-terminal end which probably serves as the transit peptide.

7.1.2. Immunolocalisation and electron microscopy

The direct *in situ* morphological localisation of nitrite reductase may be achieved by immunogold labelling of ultrathin tissue sections viewed under the electron microscope. This technique involves the incubation of ultrathin tissue sections with specific antiserum to allow antigen-antibody binding followed by labelling of the specifically bound primary antibody with either protein A or a secondary anti-IgG antiserum conjugated with gold colloids. Thus, the intracellular localisation of the antigen may be visualised, on the ultrathin sections under the electron microscope, due to the presence of the electron dense gold particles (Roth, 1984).

Electron dense particles are particularly useful for immunolabelling ultrathin sections since alternatives such as radiolabels combined with autoradiography or enzyme markers give a less precise antigen localisation and require more complicated labelling procedures which are not compatible with good preservation of ultrastructural detail. One of the first electron dense immunomarkers was the iron storage protein ferritin (Singer, 1959). However, more recently colloidal gold (Faulk and Taylor, 1971) has been used extensively in immunolocalisation studies because of its high electron density, the availability of many different particle sizes and the simple procedure by which they may be coupled to macromolecules.

Preparation of tissue for electron microscopy requires the chemical fixation, organic solvent dehydration and embedding of tissue in resin. It is widely recognised that these processes adversely affect antigenicity and appropriate conditions have to be devised for each particular antigen.

Tissue is usually fixed with various concentrations of glutaraldehyde (0.5 to 4 per cent) or mixtures of paraformaldehyde (2 to 4 per cent) and glutaraldehyde (0.1 to 2 per cent). Dehydration is performed with either ethanol or acetone before the tissue is embedded in resin.

Development of resins such as L.R. White for immunolabelling studies (Goodchild, 1985; Van den Bosch and Newcomb, 1985; Vaughn, 1987) allows immunochemicals to permeate the supporting resin, due to its hydrophilic nature, and react with tissue antigens, if they have been preserved in the tissue. Resins such as L.R. White must be thermally cured during the polymerisation step (Van den Bosch and Newcomb, 1986) which may also affect antigenicity. However, low temperature embedding procedures with specialised resins such as Lowicryl® K4M photopolymerised by ultraviolet light (Goodchild *et al.*, 1985; Kamachi *et al.*, 1987) allows the embedding of tissue containing thermally susceptible antigens.

Therefore, the technique of immunogold labelling may allow the *in situ* localisation of proteins that up to now may only have been inferred from biochemical, physiological and molecular studies. However, the procedure demands a "trial and error" approach in order to determine the optimum conditions for fixation and labelling of tissue most suited to the preservation of antigenic sites.

7.1.3. Mutant analysis

The technique may also be of use in the characterisation of mutants defective in nitrite reduction. As stated earlier many types of mutation may be deduced *a priori* from a previous knowledge of the pathway. Clearly some types of mutation affecting nitrite reduction would be characterised by an abnormal distribution of the enzyme within leaf tissue. Mutations may result in defects in chloroplast recognition, processing of the precursor protein, transport into the chloroplast and even localisation within the chloroplast itself. In such cases immunogold labelling may be the only method to provide definitive evidence for the biochemical basis of the mutation responsible for the defect in nitrite reduction.

7.2. Results

7.2.1. Leaf tissue fixation

A range of fixation protocols, 1 to 4 per cent glutaraldehyde and mixtures of glutaraldehyde and paraformaldehyde (Table 7.1) were employed to determine the optimal conditions for barley leaf tissue fixation. Tissue integrity was maintained with a fixative concentration of at least 2.5 per cent glutaraldehyde with the cellular ultrastructure well defined (Fig 7.1) in tissue post-fixed stained with osmium tetroxide.

In vitro nitrite reductase activity in leaf extracts from 7 day old plants to be fixed (treated with 25mM KNO_3 for 3 days) was almost double that found in leaf extracts from 7 day old plants treated with 25mM KNO_3 for 24 hours on a fresh weight basis (130μmoles nitrite reduced/gm fresh weight/hour (Table 7.1) compared to 67μmoles nitrite reduced/gm fresh weight/hour) whereas the specific activity increases on average by only just over 40 per cent (10.5 μmoles nitrite reduced/mg protein/hour (Table 7.1) compared to 7.9μmoles nitrite reduced/mg protein/hour (section 6.2.2.)). Thus, the increase in nitrite reductase activity is due to induction by nitrate and an overall increase in protein content in the seedlings.

7.2.2. Immunogold localisation

Nitrite reductase antiserum was used at various dilutions (neat to 1:2,000) to determine the optimum dilution of antiserum for immunogold labelling of sections of barley leaf tissue for nitrite reductase (Table 7.2). Dilutions below 1:100 resulted in a high density of label covering the entire section (Table 7.2) while dilutions greater than 1:100 resulted in a reduction in total label unless the sections were incubated for longer periods of time (Table 7.2). Thus, a primary antiserum dilution of 1:100 was chosen as the optimum for immunogold labelling which was finely tuned by adjustments made to the basic procedure involving buffer composition and method for washing grids as well as an increase in the dilution and incubation time for GAR G15 to give the final procedure described in the materials and methods (section 2.5.5.).

Using this procedure sections of barley leaf, from nitrate-grown plants, demonstrate preferential labelling of the chloroplasts after treatment with nitrite reductase antiserum (Fig 7.2). Label is not present on control sections incubated with pre-immune antiserum or when the primary antiserum was omitted (Fig 7.3).

Table 7.1. Optimisation of barley leaf tissue fixation.

fixative	nitrite reductase activity µmoles/gm/hour	µmoles/mg protein/hour
1% glutaraldehyde	120	9.3
2% glutaraldehyde	120	9.3
2.5% glutaraldehyde	133	11.5
3% glutaraldehyde	133	11.5
3.5% glutaraldehyde	118	10.0
4% glutaraldehyde	118	10.0
1% glutaraldehyde + 2% paraformaldehyde	158	11.2
2.5% glutaraldehyde + 1.5% paraformaldehyde	158	11.2

Leaf tissue from 7 day old barley seedlings (4 days germination in the dark watered with half-strength Hoaglands nutrient solution minus nitrate plus 3 days growth in the light watered with half-strength Hoaglands nutrient solution containing 25mM KNO_3) were sliced into $1mm^2$ pieces with a razor and placed in the fixative solution in 0.2M potassium phosphate buffer pH 7.3 for 1-2 hours at 4^oC. Half the tissue from each fixation procedure was then post-fix stained with 1% osmium tetroxide for 1 hour at 4^oC. Both stained and unstained tissue was then washed (2 X 15 minutes) in 0.2M potassium phosphate buffer pH 7.3 before embedding in resin. Samples of leaf tissue were removed for *in vitro* methyl viologen nitrite reductase assays immediately prior to tissue fixation.

Fig 7.1. Chemical fixation of barley leaf tissue.

Cellular integrity and organelle ultrastructure was maintained in tissue fixed with 2.5 per cent glutaraldehyde in 0.2M potassium phosphate buffer pH 7.3 for 1-2 hours. Post-fixation staining with 1% osmium tetroxide allowed visualisation of membranes and identification of cell organelles, thus enabling *in situ* localisation of gold label.

nuc: nucleus, chl: chloroplast, mit: mitochondria, vac: vacuole, cyt: cytoplasm, cw: cell wall.

Magnification = X 7,000.

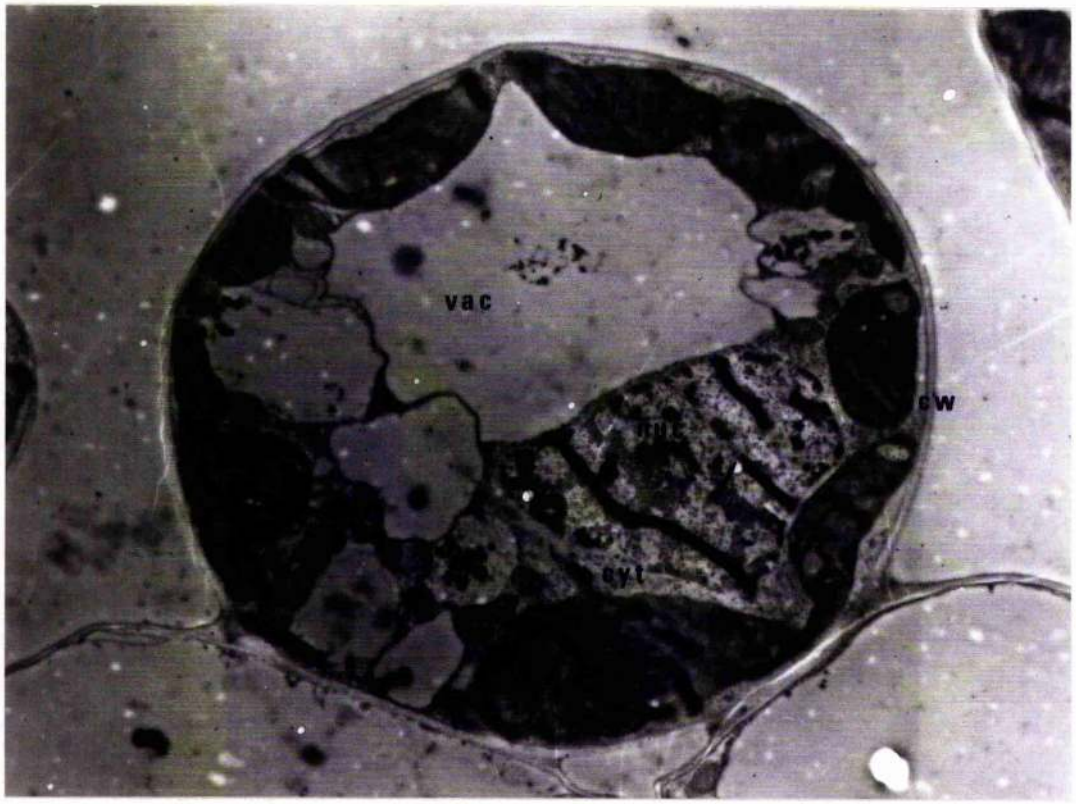

vac
cw
cyt

Table 7.2. Optimisation of immunogold labelling of barley leaf tissue sections for nitrite reductase.

primary antiserum dil^n	time (hours)	GAR G15 dil^n	time (mins)	Average label total	chlor	non-chlor
neat	1.5	1:100	30	TMTC	TMTC	TMTC
1:10	1.5	1:100	30	TMTC	TMTC	TMTC
1:100	1.5	1:100	30	75	40.5	43.5
1:1000	1.5	1:100	30	35	16.8	18.2
1:1000	5.0	1:100	30	82	33.6	48.4
1:2000	1.5	1:100	30	74	13.3	60.7

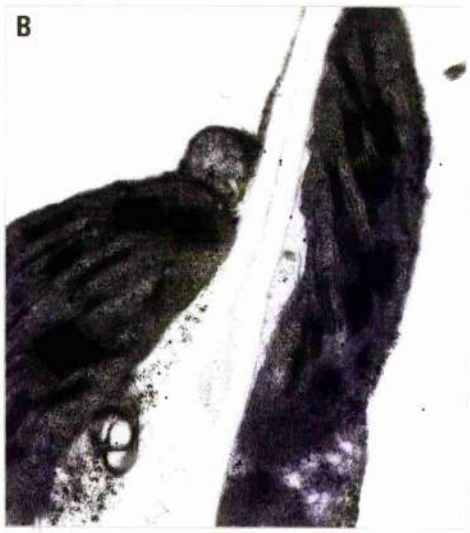
TMTC : Too many to count

Sections of leaf tissue from nitrate and light-grown barley were incubated with 1mg/ml BSA for 10 minutes, then subjected to various dilutions of nitrite reductase antiserum. The grids were then jet washed with 150ml phosphate buffer saline (PBS) followed by incubation with goat anti-rabbit IgG conjugated to 15nm gold particles (GAR G15). After jet washing the grids with 150 ml PBS, and staining with uranyl acetate and lead citrate, the sections were viewed under the electron microscope and the gold label within the chloroplasts and elsewhere within the field counted.

Fig 7.2. Nitrite reductase immunolocalisation in barley leaf.

Ultrathin sections of barley leaf fixed in glutaraldehyde, post-fix stained with osmium tetroxide and embedded in L.R. White resin were treated with nitrite reductase antiserum followed by GAR G15 and uranyl acetate and lead citrate staining. The majority of gold particles were localised within the chloroplasts, with equal labelling on the stroma and grana. Magnification: A = X 13,000, B = X 22,000, C = X 10,000 and D = X 22,000.

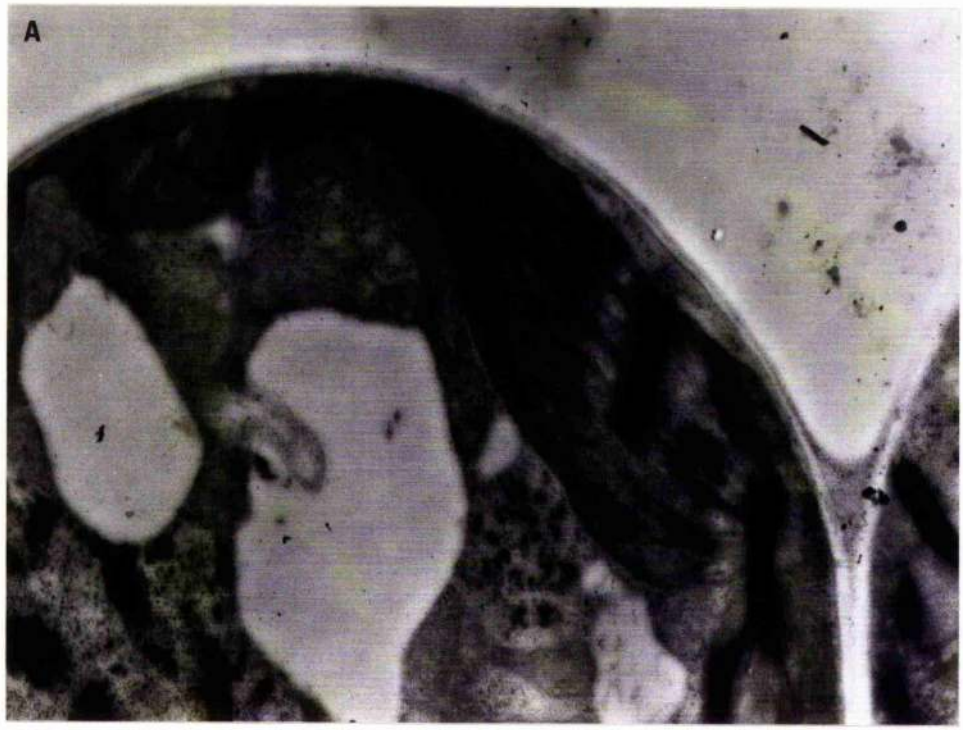

A

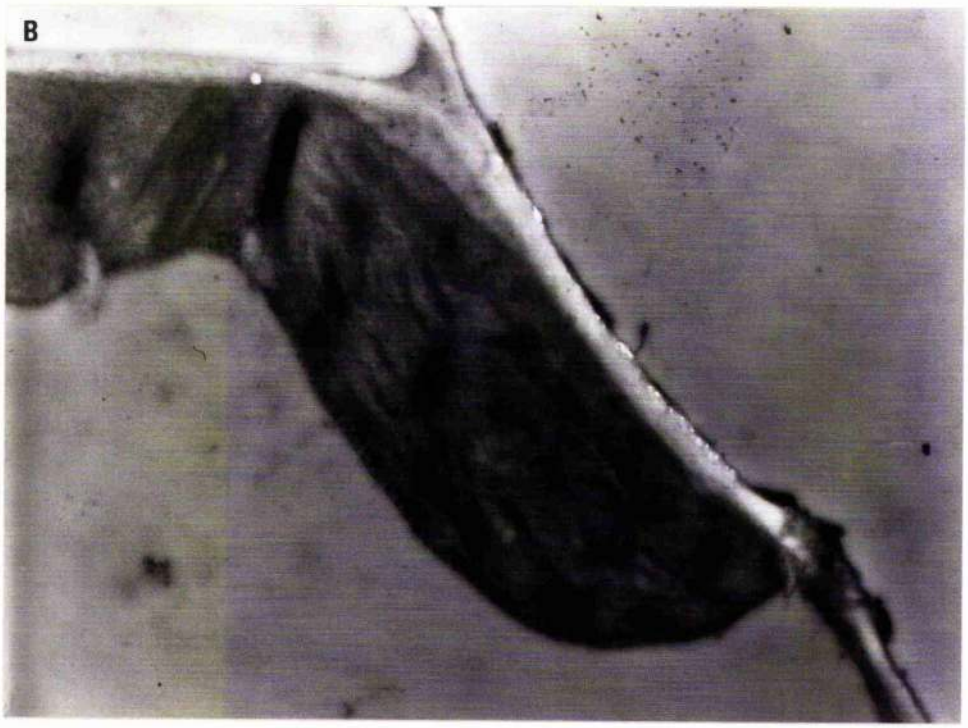

B


C

Fig 7.3. Non-specific immunolabelling of barley leaf sections for nitrite reductase.

Ultrathin sections of barley leaf fixed in glutaraldehyde, post-fix stained with osmium tetroxide and embedded in L.R. White resin were treated with either rabbit pre-immune serum followed by GAR G15 (A) or GAR G15 only (B) and stained with uranyl acetate and lead citrate. Label was absent from these sections indicating no non-specific interaction between the antiserum and the tissue sections. Magnification: A and B = X 21,000


A


B

The gold particle label density was greater over the chloroplasts compared to the other organelles (Table 7.3). In a one-tailed significance test of the difference between the mean gold particle label density for the chloroplast and each of the other organelles the chloroplast was always found to contain a significantly higher gold particle label density at a 99% level of confidence.

7.2.3. Intra-chloroplastic immunolocalisation

Immunogold label was distributed evenly within chloroplasts with granal stacks and the stromal matrix both possessing gold label (Fig 7.2 and Table 7.4).

Table 7.3. Mean label density of gold particles per μm^2 from barley leaf sections labelled for nitrite reductase.

	Number of Organelle profiles	Gold particles counted per $\mu m^2 \pm$ S.E.
Chloroplast	40	4.71 ± 0.81
Cytoplasm	42	2.08 ± 0.57
Mitochondria	31	1.07 ± 0.31
Vacuole	41	0.35 ± 0.08
Nucleus	16	0.52 ± 0.11
Cell wall	39	1.20 ± 0.27

Sections of barley leaf tissue labelled with rabbit anti-nitrite reductase IgG followed by goat anti-rabbit IgG 15nm gold complex were photographed under the electron microscope. The micrographs were then analysed for gold particle density within the various organelles present in the plant cell.

Table 7.4. Intra-chloroplastic immunolocalisation of nitrite reductase.

chloroplast ultrastructure	number of micrographs	gold particles	(%)
stroma	39	657	52.9
membrane (grana and single lamellae)	39	586	47.1

Chloroplasts within electron micrographs of barley leaf sections immunolabelled for nitrite reductase were analysed for distribution of gold label within the stroma or attached to granal stacks or single lamellae. Any label which was wholly or partly in contact with a membrane (either single lamellae or grana, but not the chloroplast envelope) was classified as membrane label. All other label within the chloroplast was classified as stroma label.

7.3. Discussion

The chloroplast is the major location for immunogold label after treatment with nitrite reductase antiserum in barley leaves as demonstrated by the preferential labelling (Fig 7.2 and Table 7.3). Although other organelles demonstrated measurable levels of label density all were significantly lower than the chloroplast label density in a one tailed test at 99% level of confidence. The labelling, both chloroplastic and elsewhere within the leaf cells, was due to the specific binding of antibodies raised against the purified barley leaf nitrite reductase by Ip *et al.*, (1987) since label was absent from control sections treated with pre-immune serum, or where the primary antiserum had been omitted (Fig 7.3).

The background label on the sections is probably due to the polyspecificity of the antiserum at low dilutions. The antiserum is monospecific for nitrite reductase when used for western blot analysis where the antiserum is used at a dilution of 1:50,000. If used at lower dilutions there is evidence for the presence of significant levels of polyspecificity (Fig 7.4). Attempts were made at reducing the amounts of polyspecific binding expressed by the antiserum by an affinity absorption procedure. Leaf tissue extract from nitrate grown barley plants separated by SDS polyacrylamide gel electrophoresis was electroblotted onto nitrocellulose filters, and the band containing nitrite reductase protein cut from the filter. The polyclonal antiserum was then incubated with the nitrocellulose filters lacking the nitrite reductase band. Any antibodies which recognise barley leaf proteins other than nitrite reductase and are thus responsible for polyspecific labelling should bind to those proteins on the nitrocellulose filter leaving anti-nitrite reductase antibodies in the antiserum. This procedure was successful in reducing polyspecific binding in western blots at low antiserum dilutions (Fig 7.5). However, recovery of anti-nitrite reductase antibodies was inefficient resulting in lower levels of nitrite reductase labelling after western blot analyses (Fig 7.5). The antiserum also gave markedly lower levels of immunogold label on leaf sections to the point where zero label was present on the sections.

Most reports of immunolocalisation do not present quantitative data on the label density within the various cellular organelles but rely on qualitatively visual data of selected electron micrographs, making direct comparisons between reports in the literature difficult. The chloroplast label density for nitrite reductase of 4.71 gold particles per μm^2 is comparable with the label densities of other enzymes reportedly located within the chloroplasts in higher plant species. Goodchild *et al.*, (1985) reported a chloroplast label

Fig 7.4. Polyspecific binding by nitrite reductase antiserum.

Antiserum raised against purified barley leaf nitrite reductase exhibits polyspecific binding with barley leaf proteins after SDS polyacrylamide gel electrophoresis followed by western blot analysis. Polyspecific binding is dependent upon the dilution of antiserum used with lower dilutions resulting in increased polyspecific binding.

1: antiserum dilution 1:100, 2: antiserum dilution 1:1,000, 3: antiserum dilution 1:10,000.

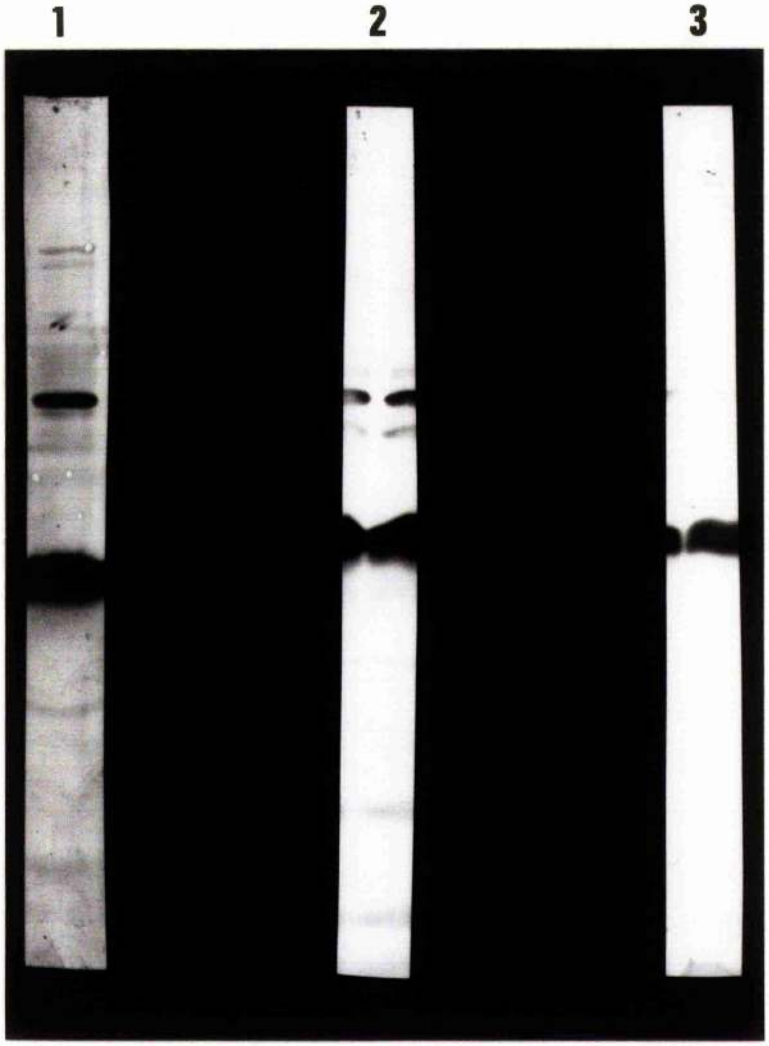
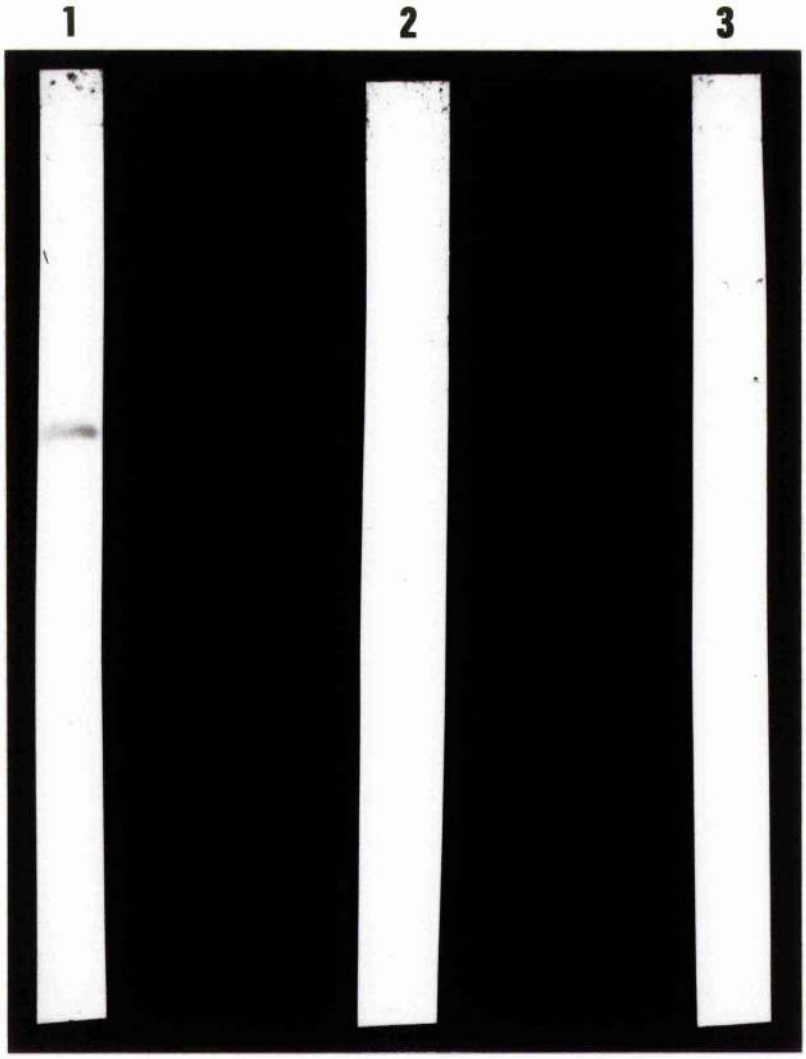

1
2
3

Fig 7.5. Affinity absorption of polyspecific nitrite reductase antiserum.

Polyclonal nitrite reductase antiserum was incubated with nitrocellulose filters (which had been electroblotted with leaf protein extract from nitrate grown plants separated by SDS polyacrylamide gel electrophoresis) after the band containing nitrite reductase had been removed. Those antibodies responsible for the polyspecificity bind to proteins carried on the nitrocellulose filters leaving the monospecific anti-nitrite reductase antibodies in the antiserum. The antiserum was collected and expressed very little polyspecific binding at low dilutions.

1: antiserum dilution 1:100, 2: antiserum dilution 1:1,000, 3: antiserum dilution 1:10,000.


1
2
3

density for the cytochrome b/f complex in spinach of 3.39 gold particles per μm^2, with a total background of 0.34 gold particles per μm^2. This background figure is misleading since the label for all the remaining organelles were averaged together to produce a diluting effect on the average label density. Indeed if the data presented here is treated in the same manner then the overall non-chloroplastic label density becomes 1.13 gold particles/μm^2, just under 25 per cent of the chloroplast label density.

Spinach nitrate reductase was controversially reported to be chloroplastic by Kamachi *et al.*, (1987) who presented micrographs demonstrating a specific labelling of the chloroplasts. However, analysis of the micrograph as performed here reveals a chloroplastic label density of 4.92 gold particles per μm^2, with the cytoplasm containing 2.82 gold particles per μm^2 and the cell wall 1.57 gold particles per μm^2. In barley leaf tissue Shaw and Henwood, (1985) presented one micrograph to demonstrate the chloroplastic location of ribulose 1,5-bisphosphate carboxylase/oxygenase. Further analysis of the micrograph to determine the label density reveals a chloroplastic label density of 11.92 gold particles per μm^2 for ribulose 1,5-bisphosphate carboxylase/oxygenase with a cytoplasmic label density of 6.33 gold particles per μm^2.

Thus, it would appear that background levels are a problem, particularly within the cytoplasm. This is partly due to the fact that the majority of the plant cell volume is made up of the vacuole which squeezes the cell organelles outwards against the cell wall, surrounded by a thin layer of cytoplasm (Fig 7.1). This produces very small areas of cytoplasm in the electron micrograph sections which tend to surround the organelles. Thus, if there is any leakage due to the fixation procedure, or mechanical contamination by the knife during sectioning, the absolute amount of label in the cytoplasm will be increased, with increases of just one or two gold particles having major effects on the mean label density due to the small areas of cytoplasm present.

Assuming average figures for the size and number of chloroplasts within an average mesophyll cell it is possible to estimate the amount of nitrite reductase protein represented by a mean chloroplastic label density of 4.71 gold particles per μm^2 (Fig 7.6). The calculation also requires assumptions to be made about the proportion of each section which is available to interact with the antibodies, without steric hindrance from the embedding resin, as well as the degree to which nitrite reductase retains its antigenic sites after fixation and embedding. It has been assumed that both processes are 10 per cent efficient thus allowing a maximum 10-fold error for each assumption which may affect the final estimation.

Fig 7.6. Theoretical limit of nitrite reductase protein in barley leaf tissue.

Assumptions made:

Chloroplasts are disc shaped bodies 5-8μm in diameter by 3μm in width

There are on average 50 chloroplasts per cell

The average mesophyll cell occupies a volume of 1×10^6 μm^3

Leaf tissue sections are 80-120nm thick

Volume available to antibody on section is 10 per cent

Retained antigenicity of protein after fixation is 10 per cent

1gm tissue is equivalent to $5cm^3$

Calculation:

A gold label density of 4.71 particles/μm^2 = 150 particles per chloroplast section

30 sections per chloroplast = 4,500 particles per chloroplast

10% of section volume available to antiserum = 45,000 particles per chloroplast

10% antigenicity retained = 450,000 particles per chloroplast

50 chloroplasts per cell = 2.25×10^7 particles per cell

5×10^6 cells per gm tissue = 1.125×10^{14} particles per gm tissue

Avogadros number 6.023×10^{23} molecules of nitrite reductase = 63kg

Therefore 1.125×10^{14} particles per gm tissue

$= 1.18 \times 10^{-8}$kg

$= 1.18 \times 10^{-5}$gm

= 11.8μg nitrite reductase per gm tissue

1gm tissue yields 10-15 mg extractable protein

Therefore nitrite reductase represents 0.1 per cent extractable leaf protein.

Using these assumptions the estimated amount of nitrite reductase represented by a gold label density of 4.71 gold particles per μm^2 is 0.1 per cent of the extractable leaf protein (Fig 7.6). This estimate is comparable with those of 0.05 per cent for nitrate reductase (Campbell, 1987) and 0.016 per cent for nitrite reductase (Ip *et al.*, 1987) after the purification of these enzymes from leaf tissue, particularly when no account has been taken of the efficiency of recovery during the purification process.

The abundance of the protein within the tissue is important, since more abundant proteins will have a greater number of antigenic sites left unaffected by the fixation process so making localisation of the protein easier. Indeed a review of the literature shows that the technique of immunolocalisation has been used far more convincingly when the protein under investigation represents a significant proportion of the protein present within the tissue, such as structural proteins (Roth *et al.*, 1983) or storage proteins (Craig and Goodchild, 1982; Sonnewald *et al.*, 1989).

Since nitrite reductase activity increases in barley leaf tissue from nitrate-grown plants due to *de novo* synthesis of nitrite reductase protein, nitrite reductase activity was used as an indication of the abundance of nitrite reductase protein molecules in leaf tissue fixed and embedded for immunolocalisation studies (Table 7.1). Leaf tissue from 7 day old plants, grown with nitrate for 3 days, possessed high levels of nitrite reductase activity, with an average of 130µmoles nitrite reduced/gm fresh weight/hour compared to 67µmoles nitrite reduced/gm fresh weight/hour for leaf tissue from plants exposed to nitrate for 24 hours, suggesting that the plants contained higher levels of nitrite reductase protein. However, the specific activity increased by just over 40 per cent (10.5µmoles nitrite reduced/mg protein/hour (Table 7.1) compared to 7.9µmoles nitrite reduced/mg protein/hour (section 6.2.2.)) indicating that the increase in nitrite reductase activity was due to both induction from prolonged exposure to nitrate and an overall increase in the protein content in these plants. Thus, the level of other protein species which may lead to non-specific immunolabelling may also be increased in these plants. Therefore, the relative abundance of nitrite reductase protein compared to other protein species may still remain at low levels.

The use of the immunolocalisation technique for the localisation of enzymes requires caution since the small proportion of enzyme molecules present in the tissue can be reduced further by the fixation process making localisation difficult. The antibodies must be monospecific at very high antibody titres to prevent the appearance of background levels

from contaminants. Monoclonal antibodies may help, although the recognition of several antigenic sites by a polyclonal antiserum can be particularly useful if some of the antigenic sites are more susceptible to denaturing by the fixation process.

The characterisation of lines selected for nitrite accumulation (chapter 4) indicated that these lines carried defects affecting the regulation and production of nitrite reductase protein. Since these lines lacked detectable nitrite reductase protein immunogold labelling experiments were not performed on the mutant material.

However, selected lines that lacked detectable nitrite reductase protein may be useful in providing control tissue for immunogold labelling experiments. Such tissue should allow the distinction between specific and non-specific labelling to be made, particularly since barley leaf tissue grown in the absence of nitrate contains detectable levels of nitrite reductase protein (chapter 6) and thus may not be used as a negative control.

Chapter 8

Discussion

8.1.1. Isolation of nitrite accumulating barley plants

This study has shown that higher plants defective in nitrite reduction can be isolated due to the expression of the unusual phenotype of *in vivo* nitrite accumulation after treatment with nitrate, as first suggested by Dunn-Coleman *et al.*, (1984) and Wray, (1986). The isolation procedure for identifying nitrite accumulators in barley is relatively labour intensive with individual nitrite accumulators selected at an overall frequency of 1:12,000.

This is somewhat lower than the frequency for nitrate reductase-deficient mutants derived from azide treated populations of barley of 1:667 (Warner *et al.*, 1977), 1:1,229 and 1:850 (Kleinhofs *et al.*, 1978) although compares with the frequency of chlorate resistant mutants, also derived from azide treated populations of barley, of 1:6,522 and 1:11,000 (Bright *et al.*, 1983).

Different loci within the barley genome will mutate, after treatment with chemical mutagens, and produce mutant individuals at different frequencies. Direct comparisons between data from different workers may be affected by inherent differences in efficiency of the mutagenic treatment. While data on chlorophyll-deficient mutants within mutagenised populations is easily collated its use as a comparative standard requires caution, since the frequency of chlorophyll-deficient seedlings expressed a negative correlation with the concentration of azide used in the mutagenic treatment on the M_1 generation (section 3.2.3.1.).

Azide may selectively interact with regions of the genome producing individuals with certain mutant phenotypes at high frequencies while others remain relatively scarce. Such specific interactions between sodium azide and different loci have been observed in *Salmonella typhimurium* (Owaiss and Kleinhofs, 1988). However, it is unknown whether sodium azide acts upon specific sequences of DNA during the mutagenic process in higher plants.

The proportion of the genome which, when carrying a mutation, may produce a mutant phenotype will also affect the frequency with which that phenotype is observed within a population. Phenotypes that may arise through a defect in one of several activities (such as chlorophyll deficiency) will require more DNA to code for those activities compared to those coded by a single gene and so are more likely to be affected by a random mutational

process. Thus, all other considerations being equal, such phenotypes should occur at higher frequencies.

Lastly certain phenotypes may not be selected since their expression would require the simultaneous mutation in two separate loci which code for the same activity. Such mutants could not realistically be selectable at the whole plant level.

The large difference in the isolation frequencies for nitrate reductase mutants in barley by screening for either a deficiency in nitrate reductase activity (Warner *et al.*, 1977: Kleinhofs *et al.*, 1978) or chlorate resistance (Bright *et al.*, 1983) demonstrates the importance of the method chosen for selecting the mutant individuals. Mutants were identified at much higher frequencies when azide-treated populations were screened for nitrate reductase deficiency compared to the more stringent screen for chlorate resistant individuals. Thus, the method of screening the mutagenised populations may, in some cases, exert a selection pressure and affect the frequency with which mutants are identified.

8.1.2. Maintenance of selections

Despite the difficulties experienced in maintaining nitrite accumulating individuals, 33 per cent of the selections were maintained to maturity and produced self pollinated seed. Maintenance of selections was complicated by various factors including microbial contamination of the hydroponic growth medium (due to the necessity of supplying the selections with a reduced nitrogen source), nitrite toxicity and weak, slow growth as a result of the azide treatment.

Recovery of 33 per cent of selections in the form of progeny seed compares favourably with the recovery of nitrate reductase-deficient mutants where 44 per cent of the selections produced progeny seed (Warner *et al.*, 1977) while only 9 per cent of chlorate resistant selections produced progeny seed (Bright *et al.*, 1983). Thus, treatment with sodium azide creates mutations at high frequencies in populations of barley to allow the selection of mutant individuals, but also appears to seriously affect the growth, survival and fertility of the selected individuals.

8.1.3. Biochemical characterisation

No biochemical data is available on the M_2 selections from the bulk-harvested populations. However, 5 lines were fertile and produced M_3 seed. The progeny from these lines did not accumulate nitrite after either *in vivo* or *in vitro* treatment with nitrate. Young M_3 seedlings possessed *in vitro* nitrite reductase activity that was about 50 per cent of the activity level measured in wild type controls due to lower levels of nitrite reductase molecules after treatment with nitrate. However, these seedlings grew vigorously in compost and possessed nitrite reductase activity levels comparable with wild type controls at a more mature stage of plant development.

Thus, these 5 fertile lines appear not to possess any obvious heritable defect in nitrite reduction to cause an accumulation of nitrite after treatment with nitrate. Thus, the 5 surviving lines selected from the bulk-harvested screen may represent false positives from the bulk-harvested screen. Mutations affecting nitrite reduction may have been carried by bulk-harvested selected M_2 plants that could not be maintained to maturity in the hydroponic growth system or were infertile.

All except one (Klaxon 48) of the chlorophyll-containing green M_2 selections analysed from the spike-harvested populations possessed the same biochemical defect (a lack of nitrite reductase protein) in nitrite reduction. Klaxon 48 may represent a false positive from the nitrite accumulation screen.

The M_3 progeny of the fertile lines (Golden Promise 2406, Tweed 3999, Klaxon 1010 and Klaxon 2760) from the spike-harvested selections inherited the defect of a lack of nitrite reductase protein. Leaf tissue extracts from the progeny plants possessed very low levels of *in vitro* methyl viologen nitrite reductase activity. The measured levels of activity were below the limit of accurate measurement but appeared to be biased towards a positive activity value. It is unlikely that the measured activity was due to a functional nitrite reductase since the leaf tissue did not possess nitrite reductase protein and possessed levels of *in vitro* methyl viologen nitrate reductase activity that was comparable to NADH-nitrate reductase activity levels. Therefore, the measured activity may be an inherent artefact of the assay due to a chemical reaction between the assay components or some unidentified biological activity which may metabolise nitrite to form some other compound.

Thus, the selections from the spike-harvested populations appear to carry defects in either the structural gene preventing formation of nitrite reductase molecules or in some regulatory loci involved in the developmental and/or environmental control of nitrite reductase. Alternatively, the mutation may affect the stability of nitrite reductase protein molecules. The immediate degradation of newly synthesised nitrite reductase molecules would lead to an apparent lack of nitrite reductase protein after western blot analysis.

The biochemical characterisation of the defect has been complicated by the small number of self pollinated seed produced by the surviving selected lines which had low germination rates, coupled with the slow, weak growth described above. This limited material was exposed to nitrate in one of two ways (either grown in vermiculite and watered with half-strength Hoaglands nutrient solution containing 25mM KNO_3 (*in vivo* induction), or was treated with nitrate *in vitro* by incubation with 0.1M KNO_3 (*in vitro* induction)) which produced conflicting results.

In vivo induction did not, in general, produce an accumulation of nitrite in leaf tissue of selected M_3 plants, while nitrite did accumulate in leaf tissue from selected M_3 (and M_2) plants subjected to *in vitro* induction. This indicates that the induction conditions are particularly important in eliciting the response of leaf tissue nitrite accumulation.

There are gross physical differences between the physiological uptake of nitrate by roots and transport throughout a whole plant (*in vivo* induction) compared to the exposure of a single piece of excised leaf tissue to nitrate by vacuum infiltration (*in vitro* induction). These gross differences between the two procedures affect the tissue nitrate content responsible for the induction of nitrate reductase, required for the formation of nitrite, and of nitrite reductase responsible for the conversion of nitrite to ammonium ions.

7 day old barley seedlings watered with nitrate (*in vivo* induction) will accumulate nitrate over the following 24 hour period to a final level of about 10nmoles nitrate/mg fresh weight tissue. However, this is a time dependent process starting from a nil value, thus the effective nitrate content may be averaged over the time period as 5nmoles nitrate/mg fresh weight tissue. This compares with *in vitro* induction where the tissue is subjected to an immediate exposure to a solution of 0.1M KNO_3 to give a final calculated tissue nitrate content of about 750nmoles nitrate/mg fresh weight, assuming nitrate equilibrates between the tissue and the incubation solution after 24 hours. Thus, the effective nitrate content may be averaged over the time period as 375nmoles nitrate/mg fresh weight. Therefore, the leaf

tissue nitrate content, responsible for the induction of nitrate and nitrite reductase is some 75-fold greater in tissue subjected to *in vitro* induction compared to *in vivo* induction. Thus, tissue from the same selected line, which will have an identical capacity to metabolise any nitrite formed, will accumulate nitrite *in vitro* since the driving force for nitrite production (the availability of nitrate to induce nitrate reductase activity and also to act as substrate for the enzyme) is at least 15 times greater in tissue exposed to nitrate by *in vitro* induction. Therefore, *in vitro* leaf tissue nitrite accumulation in M_3 selected lines should be considered a consequence of increased nitrite formation and not a decrease in nitrite metabolism.

This suggests that a steady-state balance exists within the selected lines between nitrite formation and its subsequent metabolism in the formation of other compounds. Those functions responsible for the formation of nitrite, nitrate uptake and *in vitro* nitrate reductase activity are similar to wild type levels after *in vivo* induction of selected lines, while, in Golden Promise 2406 at least, *in vivo* nitrate reductase activity is comparable to wild type after exposure to nitrate by vacuum infiltration. Thus, the formation of nitrite in selected lines should occur normally. These lines (Golden Promise 2406, Tweed 3999, Klaxon 1010 and Klaxon 2760) lack significant *in vitro* methyl viologen nitrite reductase activity due to their inability to produce detectable nitrite reductase protein in response to nitrate and light. Therefore, these lines are incapable of further metabolism of nitrite via its reduction to ammonium ions by the action of nitrite reductase. Yet these plants are able to maintain the balance between nitrite production and metabolism after *in vivo* induction, preventing any nitrite accumulation, while *in vitro* induction tips the balance in favour of nitrite formation due to an effective increase in nitrate content resulting in a net accumulation of nitrite.

It has been shown that progeny from selected lines are able to metabolise nitrite *in vivo* thus preventing any accumulation of nitrite in leaf tissue. Almost all M_3 plants (except Klaxon 1010) tested from selected lines (Golden Promise 2406, Tweed 3999 and Klaxon 2760) failed to accumulate nitrite after *in vivo* treatment with nitrate and only 50 per cent of F_2 *in vitro* nitrite accumulators derived from Tweed 3999 gave a positive test for leaf tissue *in vivo* nitrite accumulation. Furthermore, when the same *in vivo* nitrite accumulating plants were rescued from the pots of nitrate-treated vermiculite and grown for a further 24 hours in nitrate-free vermiculite they did not possess any detectable leaf tissue nitrite. Thus, these F_2 plants are, in some way, capable of metabolising or removing accumulated nitrite from their leaf tissue.

The metabolism of nitrite to prevent its accumulation after *in vivo* induction may be due to several factors. Nitrite may be reduced to ammonium ions by some small residual nitrite reductase activity. However, since nitrite reductase protein is not detectable in nitrate-treated selected lines after western blot analyses (which can be detected in wild type plants grown in the absence of nitrate) and *in vitro* methyl viologen nitrate reductase activities from selected lines are virtually identical to their *in vitro* NADH nitrate reductase activities it would appear unlikely that nitrite reductase activity is present in these lines.

Alternatively, nitrite reductase may be present in selected lines, but be altered so that the protein is unstable and is degraded during extraction for *in vitro* analysis. Thus, the selected lines appear to lack any nitrite reductase protein or *in vitro* activity. However, *in vivo* the altered nitrite reductase protein may possess limited activity that allows the metabolism of nitrite within the plant and so prevents nitrite accumulation. ^{15}N analysis on lines Golden Promise 2406 and Tweed 3999 demonstrates that the selected lines are capable of limited nitrate assimilation (10-25 per cent of the wild type controls). Since these lines possess wild type levels of nitrate uptake and nitrate reductase activity, they should be capable of producing nitrite to levels comparable with wild type. Thus, if an altered and highly unstable nitrite reductase protein is present in these lines, then it appears that the reduced activity cannot, by itself, account for the metabolism of all the nitrite produced.

Nitrite may be metabolised by the action of sulphite reductase which like nitrite reductase contains a Fe_4S_4 centre and sirohaem and is able to catalyse the ferredoxin or methyl viologen dependent six-electron reduction of sulphite to sulphide and nitrite to ammonium ions in spinach (Krueger and Siegel, 1982). Although sulphite reductase may actively metabolise nitrite *in vivo*, selected lines that lack nitrite reductase possess significant levels of methyl viologen nitrate reductase activity suggesting that sulphite reductase-linked methyl viologen nitrite reductase activity is not present, at least in leaf tissue extracts, from these selected lines.

The inability to detect nitrite in M_3 progeny leaf tissue from selected lines may result from a purely chemical reaction without the involvement of any biological activity. Nitrite is an extremely reactive compound, particularly under the mildly acidic conditions within the plant cell reacting with a number of substances (amines, amino acids, proteins, phenols, tannins, haems and indole acetic acid, Churchill and Klepper, 1979) responsible for the phytotoxic effects of accumulated tissue nitrite, produced by several herbicides. Nitrite would

no longer be detectable once it had reacted with the various cell components, although the capacity to prevent nitrite accumulation by such reactions would be limited. Chemical reactions between nitrite and proteins or amino acids within the plant cells may also be responsible for the apparent low level incorporation of $^{15}NO_3$ into proteins. Thus, the lines tested would be unable to assimilate nitrate.

The observed *in vitro* nitrite reductase activity may also be due to a purely chemical reaction. Nitrite is present in *in vitro* nitrite reductase assays at an initial concentration of 2mM. Thus, nitrite may react with components of the leaf tissue extract so decreasing the level of nitrite in comparison to the controls where the leaf tissue extract was withheld until after the addition of sulphanilamide in 3N HCl. Nitrite produced during *in vitro* methyl viologen nitrate reductase assays is unlikely to react with proteins from the leaf tissue extract since the final concentration of nitrite in the assay remains extremely low at about 10μM (0.5 per cent of the nitrite concentration in the *in vitro* nitrite reductase assay).

Lastly, nitrite metabolism may occur through the action of some, as yet unidentified biological activity. Such an activity would not necessarily be primarily involved with nitrite metabolism, but should be able to convert nitrite into some other compound in selected lines where there is no competition for nitrite by nitrite reductase.

The presence of a steady-state between the production and subsequent metabolism of nitrite thus preventing nitrite accumulation in leaf tissue could also explain the observed phenotype of selection Golden Promise 4169 V. This plant lacked detectable nitrite reductase protein and yet failed to accumulate nitrite after treatment with nitrate while 4 other seedlings carried on spike Golden Promise 4169 (GP4169 I, II, VI, VII) were positive for nitrite accumulation. Selection GP4169 V may not have accumulated nitrite since the rate of nitrite production was insufficient to saturate any activity that metabolised nitrite resulting in no net accumulation of nitrite. The other nitrite accumulating seedlings carried on the selected spike must have produced nitrite at a greater rate than that at which nitrite was metabolised resulting in a net accumulation of nitrite.

While Golden Promise 4169 V may be a single anomally, further examples, by the very nature of the phenomenon, would be difficult to find. Golden Promise 4169 V would not have been selected in the bulk-harvested screen, or indeed in the spike screen if it had not fortuitously been carried on the same spike as positive nitrite accumulators.

The inability of individual plants to saturate the steady-state level of nitrite metabolism and thus produce *in vivo* nitrite accumulation may partly explain the comparatively low frequencies with which nitrite accumulators were identified. This suggests that the screen for nitrite accumulators did not allow the selection of all the nitrite reduction-deficient seedlings contained within the screened populations. Indeed this has already been demonstrated since it is clear that the screen contained some selection pressure due to the lack of other biochemical phenotypes apart from a lack of nitrite reductase protein. The lack of such phenotypes resulting from mutations within other loci that may affect nitrite reduction (such as processing of the precursor protein; in recognition of the chloroplast/plastid; formation and insertion of prosthetic groups and in electron donation) may in some cases be due to lethal mutations, particularly those exerting pleiotropic effects. However, other types of mutation may not have been found due to the leaky nature of the mutation (either by itself or in conjunction with any inherent ability to metabolise nitrite without a functional nitrite reductase) allowing the steady-state level of tissue nitrite to remain at zero.

Individual differences in the amount of nitrate taken up and effective in inducing nitrate reductase activity in individual M_2 plants would also account for the observed differences in the amount of nitrite accumulated by selected plants. Such differences could be due to localised nitrate concentrations within the tray or perhaps due to the vigour and metabollic activity of the individual plants.

It remains unclear how the M_3 progeny of selected M_2 nitrite accumulators, in general, fail to accumulate nitrite when treated with nitrate *in vivo*. The concentration of nitrate to which the plants are exposed is important, but in each case both the M_2 selections and their M_3 progeny seedlings were watered with 25mM KNO_3. However, the growth conditions were altered in one respect, the M_2 populations screened for nitrite accumulation were grown in trays of vermiculite (38cm x 22cm x 6cm) containing up to 200 seedlings while M_3 progeny were grown separately in pots of vermiculite (5cm diameter by 5cm height). Thus, the difference in the ability to accumulate nitrite between the M_2 selections and their progeny may be due, in some way, to differences in nitrate availability to the seedlings grown in the trays compared to the individual pots.

Alternatively, the M_3 progeny may contain some physiological defect, perhaps due to poor seed formation by the M_2 parental plants in the hydroponic growth system. The M_3

seedlings may, therefore, be metabolically less active and require higher levels of nitrate to induce sufficient levels of *in vivo* nitrate reductase activity to produce an overall accumulation of nitrite.

8.1.4. Genetics of defect

The mutation responsible for leaf tissue *in vitro* nitrite accumulation due to the lack of nitrite reductase protein occurs within a single nuclear recessive locus. F_2 progeny of the F_1 plant derived from cross pollination between Tweed 3999 and Tweed wild type segregated to produce leaf tissue *in vitro* nitrite accumulators in a ratio of 27:66 which is not significantly different from the Mendelian ratio of 1:3 for a single nuclear gene. There was 100 per cent correlation between leaf tissue *in vitro* nitrite accumulation and loss of nitrite reductase protein, demonstrating that a single mutation was responsible for both phenotypes.

8.1.5. Spike screening

The theoretical basis underlying the spike screening program for identification of heterozygotes has been proved valid by the recovery of chlorophyll-deficient mutants within segregating populations of M_3 seedlings derived from self pollination of M_2 heterozygous plants carried on M_1 spikes selected for chlorophyll deficiency. The recovery of chlorophyll-deficient seedlings in the overall ratio of 1:3 chlorophyll-deficient:wild type from self pollinating normal chlorophyll-containing parents indicates that a single nuclear recessive locus was responsible for the chlorophyll-deficient phenotype. Thus, spikes carrying seed with a homozygous mutation may also carry seed with the same mutation in the heterozygous form.

Therefore, any screening procedure that reliably identifies nitrite reductase-deficient seedlings such as *in vitro* nitrite accumulation should allow the screening of the readily available library of M_3 families derived from M_1 spikes identified as carrying nitrite reductase-deficient progeny. Identification of heterozygotes would confirm the genetic basis for the nitrite reductase-deficient phenotype in those plants.

8.1.6. Root nitrite reduction

Nitrite reductase in wild type barley roots possesses several characteristics in common with the enzyme from wild type leaf tissue. The proteins share common antigenic sites and are the same molecular weight. The root enzyme can utilise dithionite reduced methyl viologen as reducing power and both root and leaf methyl viologen nitrite reductase activities comigrate in non-denaturing polyacrylamide gels. Root and leaf *in vitro* methyl viologen nitrite reductase activities respond to nitrate due to *de novo* synthesis of nitrite reductase protein but light, unlike leaf nitrite reductase, has no effect on the control of the enzyme in root tissue.

Selected lines lacking leaf nitrite reductase protein also lack nitrite reductase in root tissue suggesting that the protein is the product of the same structural gene or under the control of the same regulatory locus in both tissues.

8.1.7. Environmental control

The environmental control of nitrite reductase in barley is similar to that found in mustard cotyledons (Rajasekhar and Mohr, 1986a), pea leaves (Gupta and Beevers, 1983) and rice leaves (Ogawa and Ida, 1987) with plants grown in the absence of nitrate possessing low levels of *in vitro* nitrite reductase activity. High levels of *in vitro* nitrite reductase activity are expressed in leaf tissue extracts of barley plants treated with light and nitrate as well as pea (Gupta and Beevers, 1983), mustard (Rajasekhar and Mohr, 1986a) and rice (Ogawa and Ida, 1987). Increases in *in vitro* nitrite reductase activity are due to *de novo* synthesis of enzyme molecules in leaf tissue from barley and leaf tissue from wheat (Small and Gray, 1984), pea (Gupta and Beevers, 1984) and rice (Ogawa and Ida, 1987).

Nitrate reductase also responds to nitrate with increases in activity due to *de novo* synthesis of enzyme molecules in leaf tissue from plants treated with nitrate in the light (Somers *et al.*, 1983). Thus, the enzymes of nitrate assimilation in barley are under similar environmental control by nitrate and light.

Nitrite reductase in root tissue of barley plants is regulated differently from leaf tissue. *In vitro* nitrite reductase activity is low in root tissue extracts from barley plants grown in the absence of nitrate. However, nitrite reductase activity increases to the same extent in root tissue from plants treated with nitrate in either the light or dark due to *de*

novo synthesis of nitrite reductase protein molecules. Thus, light has no effect upon the regulation of nitrite reductase in barley roots.

8.1.8. Further work

Further analysis of mutant lines should allow a fuller characterisation of the defect, leading to a loss of nitrite reductase protein, at the biochemical, physiological and molecular level.

The *in vivo* accumulation of nitrite by plants grown with increasing concentrations of nitrate should determine whether a steady-state level of nitrite can be set up within plant leaf tissue. This may lead to an optimisation of nitrate feeding to overcome any selection pressure within the screen for *in vivo* nitrite accumulators and allow the identification of plants carrying different types of mutations in any future screening program.

Molecular analysis would allow characterisation of the defect to determine whether the *de novo* synthesis of nitrite reductase molecules in response to nitrate is affected by the mutation at the transcriptional or translational stage.

Further screening of M_3 families from selected M_1 spikes by *in vitro* nitrite accumulation may allow the identification of heterozygotes. This would confirm the genetic basis of the defect in those lines tested. The heterozygotes could then be grown in compost without the need for maintenance of lines in the labour intensive hydroponic growth system. The segregating progeny from these heterozygotes would then give large numbers of homozygous mutants for fuller analysis.

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